



Contents lists available at SciVerse ScienceDirect

Advanced Drug Delivery Reviews

journal homepage: www.elsevier.com/locate/addr

Liposomal drug delivery systems: From concept to clinical applications

Theresa M. Allen ^{a,*}, Pieter R. Cullis ^b^a Emeritus, University of Alberta, Canada^b Department of Biochemistry & Molecular Biology, University of British Columbia, Canada

ARTICLE INFO

Article history:

Received 23 May 2012

Accepted 20 September 2012

Available online xxxx

Keywords:

Liposome
Lipidic nanoparticle
Polyethylene glycol
Anti-cancer drugs
siRNA
Pharmacokinetics
Biodistribution
Ligand-targeted

ABSTRACT

The first closed bilayer phospholipid systems, called liposomes, were described in 1965 and soon were proposed as drug delivery systems. The pioneering work of countless liposome researchers over almost 5 decades led to the development of important technical advances such as remote drug loading, extrusion for homogeneous size, long-circulating (PEGylated) liposomes, triggered release liposomes, liposomes containing nucleic acid polymers, ligand-targeted liposomes and liposomes containing combinations of drugs. These advances have led to numerous clinical trials in such diverse areas as the delivery of anti-cancer, anti-fungal and antibiotic drugs, the delivery of gene medicines, and the delivery of anesthetics and anti-inflammatory drugs. A number of liposomes (lipidic nanoparticles) are on the market, and many more are in the pipeline. Lipidic nanoparticles are the first nanomedicine delivery system to make the transition from concept to clinical application, and they are now an established technology platform with considerable clinical acceptance. We can look forward to many more clinical products in the future.

© 2012 Elsevier B.V. All rights reserved.

Contents

1. Introduction: the pioneers	0
2. Designing liposomes to achieve optimized properties	0
2.1. Drug loading and control of the drug release rate	0
2.2. Overcoming the rapid clearance of liposomes	0
2.3. Intracellular delivery of drugs	0
3. Receptor-mediated endocytosis of ligand-targeted liposomes	0
3.1. Passively targeted vs. ligand-targeted liposomes	0
4. Triggered release	0
5. Delivery of nucleic acids and DNA	0
6. Combination therapy	0
7. Multi-functional, multi-component formulations	0
8. Clinical development	0
9. Conclusions	0
Acknowledgements	0
References	0

1. Introduction: the pioneers

Since the internet has made literature searches relatively straightforward, there has been a tendency to overlook the early scientific literature and to forget, or fail to cite, the important contributions of the

early pioneers in the liposome field. We have made a special effort in this paper to find those early references and give credit to the liposome pioneers — and put their contributions into context.

It is our intent to focus on the early work in the liposome field, especially work done with small molecule therapeutics, and we apologize to our many colleagues whose more recent work we have not been able to cite due to space limitations. Some of their work is described in detail in other papers in this 25th anniversary volume. We have not covered several large areas of liposomal research, including vaccines [1,2], imaging [3,4], and applications in cosmetics

* This review is part of the *Advanced Drug Delivery Reviews* theme issue on "25th Anniversary issue – Advanced Drug Delivery: Perspectives and Prospects".

* Corresponding author at: 6 Sennok Crescent, Vancouver BC, Canada V6N 2E4.

E-mail addresses: tallen@ualberta.ca (T.M. Allen), pierc@mail.ubc.ca (P.R. Cullis).

and cosmeceuticals [5,6]. The reader is directed to the cited excellent recent reviews. Recent progress in intracellular delivery [7], including mitochondrial targeting [8], and lysosomal targeting [9] is covered in the chapter by Alvarez.

In their 1965 citation classic, the late Alec Bangham and colleagues published the first description of swollen phospholipid systems [10] that established the basis for model membrane systems [11,12]. Within a few years, a variety of enclosed phospholipid bilayer structures consisting of single bilayers, initially termed ‘bangosomes’ and then ‘liposomes’ [13], were described [14], and the early pioneers such as Gregory Gregoriadis, established the concept that liposomes could entrap drugs and be used as drug delivery systems [15–18]. Other early pioneers were showing that liposomes could change the *in vivo* distribution of entrapped drugs [19–22]. At the same time, new methods were being developed to enable the preparation of large unilamellar liposomes (LUV), with improvements in trapping efficiency and homogeneity [23,24]. The production of LUV by extrusion of multilamellar vesicles through polycarbonate filters with pore sizes of 100 nm or less was a particularly important advance. As originally proposed the liposomes were formed in a low pressure, low throughput, fashion [24], and subsequently higher pressure systems were developed to achieve larger scale production [25,26]. The production of “limit size” LUV with diameters less than 50 nm was until recently only possible using sonication or homogenization [27], however microfluidic mixing techniques now allow scalable production of LUV in the 20–50 nm size range [28].

Some of the first demonstrations of the improved *in vivo* activity of liposome-entrapped drugs in animal models used the anti-cancer drug cytosine arabinoside to demonstrate significant increases in the survival times of mice bearing L1210 leukemia [29,30], and this became a popular ‘model system’ for testing the effects of a wide variety of liposome characteristics on therapeutic outcomes. Other liposomal small molecule therapeutics were also being tested *in vivo*, with improvements in disease outcomes in animal models of disease [31–35]. These experiments were to be followed by extensive studies employing liposomal amphotericin B [36] and liposomal doxorubicin [37] that ultimately led to the first clinical trials of liposomal drugs.

2. Designing liposomes to achieve optimized properties

2.1. Drug loading and control of the drug release rate

It soon became clear that there were a number of problems associated with the *in vivo* use of the 1st generation liposomes, sometimes termed ‘classical’ or conventional liposomes. A very early observation was the difficulty in retaining some types of entrapped molecules in the liposome interior [16,22,38]. Drug release was shown to be affected by exposure to serum proteins [39–41]. Changing the content of the liposome bilayer, in particular by incorporation of cholesterol [40,42,43] was shown to ‘tighten’ fluid bilayers and reduce the leakage of contents from liposomes. Switching from a fluid phase phospholipid bilayer to a solid phase bilayer also reduced leakage [44], as did incorporation of sphingomyelin into liposomes [45,46].

Choosing drugs with physical characteristics that make them amenable to retention in liposomes is another approach to controlling the release rate of entrapped substances. Similar to biological membranes, model membranes such as liposomes have low permeability to hydrophilic drugs and high permeability to hydrophobic drugs. Indeed, to this day, retention of highly hydrophobic drugs such as paclitaxel in liposomes is problematic [47,48]. A major advance in this area was the development of drug loading in response to transmembrane pH gradients that were generated in response to internal acidic buffers, or proton-generating dissociable salts such as ammonium sulfate [49–51]. This drug-loading potential was originally demonstrated for weak bases used to measure pH gradients across

membranes, and later was extended to drugs that are weak bases [52,53]. The term remote loading is often used to describe this procedure, because the drug is loaded after the vesicles are formed. The advantage of this is that the loading of the drug can be performed independent of the time and site of liposome manufacture. Many drugs in current use are weak bases possessing a primary, secondary or tertiary amine that can be loaded in response to pH gradients [54]. The retention properties of drugs in liposomes are drug dependent; drugs such as doxorubicin precipitate readily inside liposomes following accumulation and have excellent retention properties, whereas other drugs such as ciprofloxacin, which do not readily precipitate, are more difficult to retain [55]. Drug retention can be improved by loading drugs to achieve high intra-liposomal drug concentrations above their solubility limits, thus enhancing precipitation [56], or by encapsulating polyanions such as dextran sulfate [51]. Drugs that are not weak bases, such as paclitaxel, can be converted to weak base prodrugs thus allowing encapsulation and liposomal retention [57].

Drug release rates have important implications for the therapeutic activities of all types of drug delivery systems, including liposomes. It is important to keep in mind that drug entrapped in liposomes is not bioavailable; it only becomes bioavailable when it is released. Hence the ability of accumulated liposomes to increase the local bioavailable drug concentrations, and increase the therapeutic outcome, only occurs when the rate of release rate of entrapped drug from the liposomes is optimized [58,59]. The drug must be delivered to the disease site and become bioavailable at a level within its therapeutic window, and at a sufficient rate, for a sufficient period, to have optimal therapeutic activity. The activity of cell cycle-specific drugs such as vincristine can be acutely sensitive to rates of release [60,61], and it is now possible to design liposomes with release rates that are tunable to the requirements of the therapeutic application [59,62].

2.2. Overcoming the rapid clearance of liposomes

Another problem was the rapid clearance of the ‘classical’ liposomes from circulation by uptake into the cells of the mononuclear phagocyte system (MPS), predominantly in the liver and spleen [20,63,64]. Except for the treatment of diseases where there was an MPS involvement [31], the rapid uptake of liposomes into the MPS substantially reduced their distribution to other tissues of the body, and were also implicated in toxicities to the MPS organs [65–67]. Initially, attempts were made to increase the circulation half-life of ‘classical’ liposomes by MPS blockade using large pre-doses of liposomes that contained no drug (‘empty’ liposomes) [68–70].

With the recognition that long circulation half-lives were needed for uptake into non-MPS tissues, came research on the surface properties of liposomes that led to their pre-mature clearance into the MPS. Initially, modest improvements in circulation half-life were achieved through reductions in vesicle size [64,71]. The opsonization of liposomes by serum proteins was suggested as a likely mechanism for the rapid clearance of liposomes into the liver and spleen [72–74], and modifications of the membrane surface led to improvements in their circulation half-lives. Early research focused on identifying differences between plain or unmodified phospholipid membranes and biological membranes with a surface layer rich in carbohydrates. Addition of the monosialoglycoprotein GM1 to liposomes composed of egg phosphatidylcholine (egg PC), in combination with cholesterol for membrane rigidity, resulted in the first long-circulating liposomes that didn’t require MPS blockade to achieve the effect [75]. Substitution of sphingomyelin for egg PC resulted in even longer circulation half-lives, and lower uptake of liposomes into the liver [75]. The mechanism was postulated to be due to increases in the surface hydrophilicity of the liposomes imparted by the gangliosides; these long-circulating liposomes were termed ‘Stealth’ liposomes [76], a

term subsequently adopted to apply to liposomes sterically stabilized with polymers such as polyethylene glycol (PEG) (see below).

Previous research by Abuchowski and McCoy on attaching PEG to proteins to increase their circulation half-life [77] pointed the way towards a simpler way of increasing the circulation half-life of liposomes. Within a few months, several papers had appeared that showed that grafting of PEG to the liposome surface resulted in substantial reductions in the rapid clearance of liposomes into the MPS [78–83], and, unlike 'classical' liposomes, the PEG-liposomes (Stealth liposomes) have dose-independent pharmacokinetics [83,84] except at very low doses where accelerated clearance (the ABC phenomenon) has been observed [85]. The demonstration of improvements in the therapeutic outcomes of Stealth liposomes relative to 'classical' liposomes in animal models of disease soon followed for a variety of therapeutics [86–91], and the first human studies demonstrating long circulation of a Stealth formulation of doxorubicin were published [92]. Shortly thereafter, the first clinical trial results using PEG-liposomes as carriers of doxorubicin were published for the treatment of Kaposi's sarcoma in HIV patients [93].

2.3. Intracellular delivery of drugs

The third problem with liposomal drug delivery is how to deliver molecules across cell membranes to intracellular sites of action. Hydrophobic weak base drugs such as doxorubicin or vincristine can enter cells as free drugs by passive diffusion down their concentration gradient in the uncharged form, while small hydrophilic drugs can use cell membrane transporters, (e.g., cytosine arabinoside can enter cells via the nucleoside transporter). Hence, passive delivery via the circulation (or local application) of liposomal small molecule therapeutics to diseased tissues, with release of the drug in the free (bioavailable) form at or near its intended site of action, at levels that exceed the minimal therapeutic concentration will result in activity.

However, many drugs, including a substantial percentage of the newer classes of therapeutics, cannot cross cell membranes to gain access to their intracellular site of action without some modifications to the basic liposomal delivery system. Certain types of endocytic cells, e.g., macrophages, will naturally endocytose liposomes into the cell interior [94]. Also, some types of membrane active liposomes, e.g., those containing fusogenic lipids or membrane active peptides, have been suggested to fuse with, or otherwise disrupt the cell membrane to result in the cytoplasmic delivery of the drug cargo [95–99], but this approach has not been widely adapted. Receptor-mediated endocytosis of ligand-targeted liposomes and their contents into the endosomal-lysosomal compartment is a popular way of introducing molecules into the cell interior, so long as the therapeutic molecule is capable of surviving the acidic and enzyme rich environment of the endosomes and lysosomes (Section 3, below).

3. Receptor-mediated endocytosis of ligand-targeted liposomes

Early in the history of liposomes it was recognized that a means of increasing the selectivity of the interaction of liposomes with diseased cells was desirable. If this interaction triggered receptor-mediated endocytosis of the liposome and its cargo into the desired cellular target, then so much the better. Antibodies were used in early experiments to mediate their specific attachment target cells [100,101], and receptor-mediated endocytosis of liposomes was demonstrated [102–104]. At the same time, new methodologies were being developed for attaching antibodies to liposomes [105,106]. Soon it was shown that antibody-targeted liposomes could improve the selective toxicity of liposomal anticancer agents to cultured cells [107]. However, antibody-targeted liposomes were rapidly cleared from circulation [108], limiting their in vivo distribution to non-MPS tissues. Nevertheless, some in vivo uptake of liposomes could be

demonstrated if the target cells were rapidly accessible from the circulation [109].

After the development of long-circulating (PEGylated) liposomes, it became apparent that, when antibodies were attached at the liposome surface, their antigen binding was masked by the presence of PEG in the same liposomes, particularly longer chain PEG molecules [110–112], although some accumulation of these liposomes could be demonstrated at target sites easily accessible from the circulation [113]. Newer coupling methods were developed that involved the attachment of antibodies, their fragments, and other ligands to the terminus of PEG molecules grafted to the liposome surface [112,114–120]. In one early example, this resulted in improved in vivo survival in animal lung tumor model relative to non-targeted liposomal drugs [121].

Overall, the methods for producing ligand-targeted liposomes are tedious, difficult to control, and lead to poorly defined systems that are often rapidly cleared from the circulation. The 'post-insertion' technique was developed to address these concerns. In this technique, micelles formed from PEG-linked ligands are incubated with pre-formed, drug-loaded, non-targeted liposomes (including commercial preparations) to convert them into ligand-targeted liposomes [122–124]. The technique is proving to have wide applicability for introducing a variety of substances onto the liposome surface.

3.1. Passively targeted vs. ligand-targeted liposomes

Much experimentation has gone into trying to understand what advantages, if any, ligand-targeted liposomes have over passively targeted (i.e., 'non'-targeted) liposomes, and what might be the appropriate clinical applications. In some reports, improvements in survival were seen for ligand-targeted liposomes compared to passively targeted liposomes [125,126], while in other cases no improvements in survival were seen [127,128]. Both targeted and passively targeted liposomes are distributed to target cells via the same passive distribution mechanism. Hence, when passively targeted and ligand-targeted liposomes have similar circulation half-lives, ligand-mediated targeting did not increase the distribution of liposomes to target tissues compared to passively targeted liposomes [128–130]. So any improvements in survival are not due to increased uptake of targeted liposomes by the diseased tissue, per se, but by increased receptor-mediated uptake of liposomes, containing entrapped drug, by the target cells [129]. Premature loss of liposome contents prior to binding and uptake results in no increased anti-tumor effect [127], another example of the importance of drug release rate to therapeutic effects (see Section 2.1).

The field of ligand-targeted liposomes has expanded rapidly and many experiments have shed light on some of the factors involved in the successes and failures of ligand-targeted vs. passively targeted liposomes. The basic principles that have evolved from the literature, derived primarily from studies in animal tumor models, are outlined below. Many of these principles apply to nanoparticles, in general, and are not limited to liposomes. The reader is referred to a number of comprehensive reviews on the topic for additional references [131–135].

- All particles reach target site (e.g., tumor) via passive targeting, and adding ligands to the particles does not increase the amount that reaches the target [128,130]
- For liposomes to localize to and bind to cells in solid tumors, a number of anatomical and physiological barriers, which vary with tumor type and location, need to be overcome before liposomes can be taken up into the cells. Also, tumor penetrability is highly heterogeneous [133,136] and is also dependent on particle size [137–139].
- For ligand-targeted, as well as passively targeted, liposomes, content retention and appropriate release rate is critical to therapeutic outcome [59,62]
- Intracellular delivery is a requirement for therapeutic activity for macromolecules (large, charged molecules, e.g., siRNA, peptides) that don't enter cells on their own [133]. Internalization can be

mediated by including antibodies or other ligands against internalizing antigens at the liposome surface, or via incorporation of fusogenic agents (lipid, peptides, etc.) into the particles.

- Ligand-mediated targeting increases the uptake of particles by the target cells themselves, depending on factors such as: vasculature permeability [136]; tumor penetrability [136]; antigen density [126]; ligand affinity [140]; binding site barrier [141].
- Multi-valent display of ligands on nanoparticles (high avidity) results in high binding avidity [142].
- Low affinity/avidity ligands bound to liposomes may have better penetrability than high affinity ligands [140,143].
- Ligand-mediated targeting has best therapeutic effects for targets that are readily accessible (no 'binding site barrier'): tumor vasculature [144]; micrometastases [145]; hematological malignancies [125], and ligand-target interactions that result in liposome internalization [146].
- For many targets, ligand-mediated targeting of liposomes will result in little or no therapeutic improvement over 'passive' targeting [127,128] (due to non-internalization, premature contents leakage, poor penetrability, low antigen density and/or 'binding site barriers').
- Targeting efficiency is related to receptor density at the cell surface [126]. The apparent receptor density at the cell surface can be increased by combining targeting agents that bind to combinations of ligands [147].
- The development costs (manufacturing, source of good such as antibodies, quality control, intellectual property) for targeted nanomedicines are much higher than those traditionally seen for small molecule therapeutics and for passively targeted liposomes [133].

Although a number of non-targeted liposomes have reached the clinic or are in clinical trials (Section 7), few targeted formulations have progressed into the clinic. A transferrin-targeted liposomal oxaliplatin formulation [148] has progressed to Phase II clinical trials, and a liposomal doxorubicin formulation targeted via anti-ErbB2-scFv formulation (MM-302) [149] has progressed to Phase I clinical trials. A transferrin-targeted lipid-based nanocomplex containing the p53 gene [150] has completed a phase I trial (E. Chang, personal communication). The slow progress to the clinic is related to the higher development costs (manufacturing, source of good such as antibodies, quality control, intellectual property) for targeted nanomedicines compared to those traditionally seen for small molecule therapeutics and for passively targeted liposomes [133], and the perception that there is 'not enough bang for the buck' for the targeted formulations. To offset the higher development costs, the therapeutic outcomes need to be considerably higher than those currently observed relative to non-targeted liposomes.

4. Triggered release

Stability of liposomes in the circulation with retention of their contents has long been recognized as a desirable liposome characteristic for successful drug delivery to diseased tissues. Over two decades ago, it was also recognized that being able to trigger the release of liposomal contents once they reached the target site would lead to improvements in therapeutic outcomes. Two main types of triggers have been explored, remote triggers such as heat, ultrasound and light, and local triggers that are intrinsic to the disease site or cellular organelles such as enzymes and pH changes. A thorough review of triggered release liposomes has recently been published [151].

The first trigger for drug release was hyperthermia (remote trigger); delivery of liposomal methotrexate was demonstrated to be four-fold higher in heated tumors versus non-heated control tumors [152]. Shortly after, pH-sensitive liposomes were formulated with the lipid palmitoyl homocysteine and their utility in increasing drug release in regions of mildly acid pH such as primary tumors or site of inflammation was proposed (local trigger) [153]. In two separate

experiments, a microwave device, or an ultrasound apparatus was used to apply hyperthermia to PEGylated liposomal doxorubicin in two different murine solid tumor models, resulting increase tumor drug concentrations and increased antitumor efficacies [154,155].

Ligand-targeted liposomes that promote internalization of the drug package into the target cell interior can be designed to release their contents in the enzyme rich, low pH environment of endosome and lysosomes through the use of pH-triggered approaches [156–159]. Liposomes can also be designed to release their contents through the use of lipids or peptides that facilitate fusion with the target cell membrane [156,160].

Enzyme-triggered release of liposome contents has also been studied for a variety of different enzymes including: phospholipase C [161,162], phospholipase A₂ [163], alkaline phosphatase [164], and matrix metalloproteinases [165]. In some cases the enzyme-triggered release has been used as a basis for immunoassays [162,164], and in other cases to release liposomal contents in the local environment of some cancers that are rich in secretory phospholipase A₂ [163,166] or matrix metalloproteinases [167]. Antibody-directed enzyme prodrug therapy (ADEPT) uses a different approach, which relies on the activation prodrugs at the disease site by pre-targeted antibody-linked enzymes [168].

Other recent advances in remote-triggered release systems include the use of ultrasound to trigger drug release from echogenic ("bubble") liposomes [169,170]; the use of light as a trigger in photosensitive liposomes [171,172]; and magnetically responsive liposomes [173], combined with hyperthermia-induced drug release [174] and most recently combined with ligand-mediated targeting [175]. Hyperthermia-triggered intracellular delivery has recently been described for Her2 affibody-targeted liposomal doxorubicin [176]. Synergy between thermal ablation and liposomal anticancer drugs has recently been described [177,178].

In general, triggered release approaches, although promising in concept, have been disappointing in practice. Two products have progressed to clinical trials [179,180], but the ADEPT approach, although showing some promise in animal tumor models [181], has been hampered by immune reactions to the enzymes in humans [182].

The most advanced application of the triggered release approaches to date seem to be those based on hyperthermia and ThermoDox®, a liposomal doxorubicin formulation that releases drug in response to a mild hyperthermic trigger [183]. ThermoDox® is in pivotal Phase III clinical trials for hepatocellular carcinoma in combination with radiofrequency ablation (RFA), in Phase II trials for colorectal liver metastases in combination with RFA, and in Phase I in women with locally recurrent breast cancer [184].

A concern to keep in mind for triggered release systems in cancer, in particular those that rely on remote triggers, is that patients rarely die of their primary tumors; many primary tumors can be surgically removed or ablated with radiation. Metastatic disease is a common cause of death in advanced cancer, but small metastatic tumors are not accessible via remote triggers. Hence, applications for remote triggered formulations should be carefully chosen and would include such applications as locally advanced disease and cancer where tissue sparing is preferred for reasons of preserving quality of life.

5. Delivery of nucleic acids and DNA

Soon after the first animal experiments began to show improved therapeutic outcomes for small molecule therapeutics, came the realization that liposomes could also be effective delivery systems for DNA [185,186], and for nucleic acid-based therapeutics such as anti-sense oligonucleotides (asODN) and siRNA [187]. In vivo delivery of polynucleic acids using lipid-based systems began with an early report that a liposomally encapsulated plasmid for rat insulin could result in gene expression following intravenous injection [188], and an early Phase I clinical trial for liposomal c-raf-1 asODN [189]. This

was followed by the demonstration by Felgner and others that fusogenic cationic lipids could be complexed with plasmid, and facilitate efficient transfection of cells *in vitro* [190–193]. An explosion of studies then followed, to exploit the potential of gene therapy both *in vitro* and *in vivo*. Despite intensive effort, however, and the synthesis of hundreds of different cationic lipids [193–195], gene expression could only be observed following local, as opposed to systemic injection, and the toxic side effects of cationic lipids became increasingly evident [196,197]. Other issues were the large size of the cationic lipid-DNA complexes and the high surface charge of these systems, which combine to result in rapid clearance from the circulation.

Drawing from experience with the delivery of small molecule anticancer drugs, attempts were then made to encapsulate plasmids in liposomal systems with small sizes and low surface charge [198], using detergent dialysis procedures and low levels of cationic lipids. Such systems could exhibit the long circulation lifetimes required to access disease sites such as tumors [199], but exhibited low encapsulation efficiencies and low levels of transfection. This was followed by attempts to generate long-circulating systems with reduced surface charge by employing ionizable cationic lipids with pKa values for the cationic moiety of 7 or lower [200,201]. This allowed encapsulation of the negatively charged nucleic acids at low pH values (e.g., pH 4), where the lipid had a positive charge, but resulted in substantially longer circulation half-lives than non-ionizable cationic lipids at physiological pH values, where the surface charge was low [201]. This process was first applied to antisense oligonucleotides [200,202] using a variation of the Batzri and Korn ethanol injection method [14]. This procedure involved making preformed vesicles at pH 4 in the presence of 40% ethanol and subsequently adding anti-sense, again in 40% ethanol pH 4, to achieve association. It was found that in order to achieve systems with small diameters, PEG-lipids were required, and optimum encapsulation was observed when DSPC and cholesterol was present [200]. Although long-circulating systems were achieved, little evidence of gene silencing could be observed and the main application was for immunostimulatory applications [1,203–206]. Using a similar lipid composition (ionizable cationic lipids, PEG-lipids, cholesterol and DSPC) other workers then used the Batzri and Korn method with improved mixing achieved by a T-tube mixer to encapsulate siRNA [207] and observed siRNA-induced gene silencing in liver (hepatocytes) following intravenous injection at dose levels of 1 mg siRNA/kg body weight [208].

The observation of hepatocyte gene silencing stimulated considerable efforts to determine the mechanism of action and to develop more potent LNP siRNA systems. These efforts have proven remarkably successful. In particular it has been shown that improvements in potency by more than two orders of magnitude can be achieved by employing ionizable cationic lipids with maximized ability to induce non-bilayer structure and with pKa values near 6.5 [209]. Further, it has been shown that the potency of these systems arises in part due to the association of apolipoprotein E with the LNP siRNA system *in vivo*, which stimulates uptake into hepatocytes via the scavenging receptor and LDL receptors [210]. This is consistent with earlier observations that receptor-mediated internalization of the ligand-bearing liposomes along with their DNA [211] or nucleic acid cargos [212] led to substantial improvements in gene expression or target knockdown compared to non-targeted systems. The new LNP siRNA made with “next generation” ionizable cationic lipids showed very good *in vivo* knockdown in rodent liver using the Factor VII assay [209,213] at dose levels as low as 0.01 mg siRNA/kg body weight.

In recent years, activity in the area of delivery of asODN, siRNA, dsRNA and microRNA has intensified [214,215], and the first clinical trials have begun (see Table 1, below). The new LNP siRNA systems have been taken into the clinic to silence PCSK9, a gene expressed primarily in hepatocytes that modulates low density lipoprotein (LDL) levels in the circulation, resulting in rapid and dramatic lowering

of LDL levels with no indication of toxicity. Also in the clinic, are LNP siRNA to silence transthyretin (TTR), for the treatment of TTR-induced amyloidosis, again resulting in dramatic lowering of TTR levels in the blood [216].

A recent advance for manufacture of LNP siRNA systems has been the application of microfluidic mixing to formulate the particles. This technique has been shown to allow highly efficient siRNA encapsulation and remarkable control of LNP size over the 20–100 nm diameter size range, with excellent *in vivo* gene silencing capabilities [217].

- The demonstration of nucleic acid activity in extra-hepatic tissues has been a challenge. One of the first examples of extra-hepatic targeting used a formulation of long-circulating cationic liposomes (CCL) [218], entrapping c-myb asODNs and targeted against the ganglioside GD2 [219], to significantly inhibit tumor growth and metastases in murine models of melanoma [220] and neuroblastoma [221]. Lung-targeted delivery of asODN and siRNA with knockdown of survivin was also demonstrated [222]. Extra-hepatic knockdown of ALK kinase in a neuroblastoma animal model, using anti-GD2 targeted long-circulating cationic liposomes (CCL) encapsulating ALK-specific siRNA, was recently reported, with substantial increases in life-span for the targeted CCL compared to non-targeted CCL or free siRNA [223,224]. Silencing of genes in immune cells such as macrophages and dendritic cells has been observed following i.v. administration of LNP siRNA systems [201]. LNP siRNA systems for silencing genes in hepatocytes, following intravenous administration, have achieved clinical validation exhibiting dramatic silencing of target genes in association with therapeutic indices of 100 or higher.

A number of general principles have emerged from the large and rapidly growing literature in the field of nucleic acid delivery:

- Positive charge, e.g., cationic lipid, is needed for efficient association of nucleic acids with lipids [190]
- A positive charge on liposomes results in their rapid elimination by the MPS and non-specific cell binding [225]
- To increase the circulation half-life of liposomal nucleic acids, they should have a near-neutral surface charge: two approaches have been used to achieve this, the formation of coated cationic liposomes (CCLs) [226] and the use of ionizable lipids [200,202,209,227]
- Ligands are needed for specific binding and internalization [218]. The ability of LNP siRNA systems to transfect hepatocytes efficiently following intravenous administration relies on association with Apo E *in vivo*, leading to uptake via the scavenging receptor on hepatocytes [210]
- Efficient endosomal release following internalization is needed for therapeutic activity [201], and this can be provided by ionizable cationic lipids with optimized bilayer destabilizing capacities and pKa [209,213].

6. Combination therapy

The principles of combination chemotherapy, i.e., the combination of therapies with different mechanisms of action and non-overlapping side effects, can be applied to the development of nanomedicines [228–234]. A variety of different types of combinations have been used in recent years, with at least additive increases in therapeutic outcomes for the combinations compared to individual therapies. Several different types of therapeutic combinations have been used including:

- Combinations of different small molecule therapeutics [230,232,234].
- Combinations involving one or two different liposomal drugs targeted against two or more different antigens on the same cells, or on two or more different types of cells [147,229,233,235,236]
- Combinations of free or liposomal asODNs or siRNAs that sensitize cells to small molecule therapeutics [228,231,237,238]
- Combining ligand-targeted particles with a remote triggered method to increase transfection [239]

Table 1

Marketed liposomal and lipid-based products, plus a selection of products in clinical development.

Product	Drug	Indications	Year approved	Reference
<i>Approved products</i>				
AmBisome (Gilead)	Amphotericin B	Fungal infections Leishmaniasis,	1990 (Europe), 1997 (USA), 2000	[255,256]
Doxil/Caelyx (Johnson & Johnson)	Doxorubicin	Kaposi's sarcoma Ovarian cancer Breast Cancer Multiple myeloma + Velcade	1995 1999 2003 (Europe, Canada) 2007	[93,257–259]
DaunoXome (Galen)	Daunorubicin	Kaposi's sarcoma	1996 (Europe), 1996 (USA)	[260]
Myocet (Cephalon)	Doxorubicin	Breast cancer + cyclophosphamide	2000 (Europe)	[261]
Amphotec (Intermune)	Amphotericin B	Invasive aspergillosis	1996	[262]
Abelcet (Enzon)	Amphotericin B	Aspergillosis	1995	[263]
Visudyne (QLT)	Verteporfin	Wet macular degeneration	2000 (USA), 2003 (Japan)	[250]
DepoDur (Pacira)	Morphine sulfate	Pain following surgery	2004	[264]
DepoCyt (Pacira)	Cytosine Arabinoside	Lymphomatous meningitis	1999	[265,266]
Diprivan (AstraZeneca)	Propofol	Neoplastic meningitis		
Estrasorb (King)	Estrogen	Anesthesia	1986	[267]
Lipo-Dox (Taiwan Liposome)	Doxorubicin	Menopausal therapy	2003	[268]
Marqibo (Talon)	Vincristine	Kaposi's sarcoma, breast and ovarian cancer	2001 (Taiwan)	[269]
Acute lymphoblastic leukemia			2012 (USA)	[270,271]
<i>Products in clinical trials</i>				
SPI-077 (Alza)	Cis-platin	Solid tumors	Phase II (development terminated)	[272,273]
CPX-351 (Celator)	Cytarabine:daunorubicin	Acute myeloid leukemia	Phase II	[274]
CPX-1 (Celator)	Irinotecan HCl:flouxuridine	Colorectal cancer	Phase II	[232,275]
MM-398 (Merrimack)	CPT-11	Gastric and pancreatic cancer	Phase II	
MM-302 (Merrimack)	ErbB2/ErbB3-targeted doxorubicin	Glioma and colon cancer	Phase I	[51]
MBP-436 (Meibopharm)	Transferrin-targeted oxaliplatin	ErbB2-positive breast cancer	Phase I	[276]
Brakiva (Talon)	Topotecan	Gastric cancer and gastro-esophageal junction	Phase II	[148]
Alocrest (Talon)	Vinorelbine	Relapsed solid tumors	Phase I	[277]
Lipoplatin (Regulon)	cisplatin	Newly diagnosed or relapsed solid tumors	Phase I	[278]
L-annamycin (Callisto)	Annamycin	Non-small cell lung cancer	Phase III	[279,280]
Adult relapsed ALL		Adult relapsed ALL	Phase I	[281,282]
Pediatric relapsed ALL and acute myelogenous leukemia		Pediatric relapsed ALL and acute myelogenous leukemia	Phase I	
Doxorubicin-resistant breast cancer		Doxorubicin-resistant breast cancer	Phase II (development terminated)	
ThermoDox (Celsion)	Thermosensitive doxorubicin	Primary hepatocellular carcinoma	Phase III	[283,284]
		Refractory chest wall breast cancer	Phase II	
		Colorectal liver metastases	Phase II	
Endo-Tag-1 (Medigene)	Cationic liposomal paclitaxel	Pancreatic cancer	Phase II	[285]
ALN-TTR ALN-PCS ALN-VSP (Alnylam)	siRNA targeting transthyretin (TTR) siRNA targeting PCSK9 RNAi targeting liver cancer	Triple negative breast cancer	Phase II	
		TTR amyloidosis	Phase I	[209,213]
		Hypercholesterolemia	Phase I	
TKM-PLK1 TKM-ApoB (Tekmira)	RNAi targeting polo-like kinase 1 (POLO)	Liver cancer and liver metastases	Phase I	
Stimuvax (Oncotherapy/Merck)	RNAi targeting apoB	Liver tumors	Phase I	[286]
Epxarel (Pacira)	Anti-MUC1 cancer vaccine	High levels of LDL cholesterol	Phase I	
Bupivacaine	Nerve block	Non-small cell lung cancer	Phase III	[287]
		Epidural	Phase II	
			Phase I	[288]

Two sets of liposomal drug combinations have entered clinical trials (**Table 1**). Two small molecules combinations are in Phase II clinical trials, CPX-351 (cytarabine:daunorubicin) in patients with newly diagnosed acute myeloid leukemia (AML) and first relapse AML [240], and CPX-1 (irinotecan HCl:flouxuridine) in patients with colorectal cancer [241].

7. Multi-functional, multi-component formulations

Increasingly, the formulation and use of multi-functional, multi-component liposomal nanoparticles, sometimes referred to as theragnostics, is being explored – formulations that carry within an individual lipidic nanoparticle functions such as site-specific targeting, biomarker and imaging capabilities, delivery of combinations of therapeutics, and response to external or internal triggers to control drug release [242]. As the complexity of lipidic nanoparticles

increases, so do the expenses and difficulties associated with their manufacture, quality control, and control over the intellectual property. To recompense for the additional expense, the gains in therapeutic benefits must be substantial. Multi-functional formulations that show only marginal clinical benefits are unlikely to be successful.

8. Clinical development

Both 'classical' and 'Stealth' liposomes have entered the mainstream as sustained release drug delivery systems [243] for the in vivo delivery of everything from small molecule therapeutics to nucleic acids. Early papers that were important in the clinical development of liposomes include a 1985 paper by Morgan et al. that demonstrated accumulation of liposomes labeled with technetium 111 in sites of infection and inflammation in humans [244], and a subsequent paper that showed accumulation of indium 111-labeled

liposomes in solid tumors [245], including Kaposi's sarcoma and malignant lymphoma [246]. These studies are the first demonstrations that liposomes can accumulate in regions of enhanced vascular permeability in humans. This effect was termed the enhanced permeability and retention (EPR) effect by the Maeda laboratory during the clinical development of the products SMANCS, a polymer conjugate [247]. Long circulating (PEGylated) liposomes were shown to have extensive accumulation in Kaposi's sarcoma and head and neck cancers, with intermediate accumulation in lung cancer and lower accumulation in breast cancer in an initial study using small numbers of patients [248]. However, to draw specific conclusions about the relationship between tumor types, stage of tumor development and liposome accumulation these studies need to be repeated with larger cohorts. The extent of liposomal accumulation may be related, at least in part, to the degree of angiogenesis of the tissues.

A number of products are on the market, with many more in clinical development (Table 1). AmBisome® and Doxil®, in particular, have both achieved considerable clinical success, with sales in the hundreds of millions of dollars per year. Although many routes of administration have been used for liposomal and lipid-based products, parenteral administration is the predominant one for clinically approved products, in particular intravenous administration. Other routes that have achieved clinical success are the ocular route [249], and the clinical product Visudyne® [250], and the transdermal routes [251,252]. Oral delivery is not generally used for liposomal products as GI degradation of the carrier results in poor bioavailability of associated drugs. Delivery to the brain after parenteral administration is generally low, although the recent use of convection and retro-convection enhanced delivery is showing some potential [253,254]. The most recent liposomal drug to receive FDA approval is Marqibo®, a liposomal formulation of vincristine that was approved in August 2012 to treat acute lymphoblastic leukemia at second or greater relapse.

9. Conclusions

In the 40 plus years from the concept of the clinical utility of liposomes to their recognized position in the mainstream of drug delivery systems, the path has been long and winding. They have been explored in the clinic for applications as diverse as imaging tumors and sites of infection, for vaccine and gene medicine delivery, for treatment of infections and for cancer treatment, for lung disease and for skin conditions. In clinical applications, liposomal drugs have been proven to be most useful for their ability to "passively" accumulate at sites of increased vasculature permeability, when their average diameter is in the ultrafilterable range (<200 nm in diameter), and for their ability to reduce the side effects of the encapsulated drugs relative to free drugs. This has resulted in an overall increase in therapeutic index, which measures efficacy over toxicity. However, the gains in therapeutic index have been more on the side of reduced toxicity than on the side of increased efficacy. Liposomes have poor extravasation into tissues with tight endothelial junctions, and this can result in a significant reduction in the side effects of the liposomal drug compared to the free (i.e., unentrapped) drug. An excellent example is the significant reduction in the irreversible cardiotoxicity of free doxorubicin when the drug is entrapped in liposomes [261,289,290]. Most drug toxicities are reduced when they are entrapped in liposomes and the only instances in which an increase in toxicity has been noted clinically are the appearance of mucositis and the increase in a reversible form of skin toxicity called palmar plantar erythrodysesthesia (PPE) (which has been also been described for some prolonged free drug infusions [291]), when long-circulating liposomal anthracyclines are given [292]. Liposomal drug delivery has become an established technology platform and has gained considerable clinical acceptance. We can look forward to many more clinical products based on small molecule drugs in the future.

The recent remarkable success of LNP formulations of siRNA in the clinic for silencing genes in hepatocytes also indicates that the successes achieved with small molecule drugs is likely to be matched for delivery of genetic drugs such as antisense, siRNA and plasmids for gene therapy applications. This success can be attributed in part to the remarkable flexibility of lipid-based delivery systems, which can efficiently encapsulate both small molecules and macromolecules, can be readily biodegradable and are biocompatible, can be manufactured in sizes down to 20 μm in diameter, can payout active agents at therapeutically optimized rates, and can interact with membrane components in a predictable manner. These properties allow a rational design approach to achieve therapeutic objectives. The fact that all issues associated with scale-up, stability, and satisfying regulatory demands have also been successfully addressed points to a plethora of new and increasingly sophisticated lipid-based therapeutics in the future.

Acknowledgements

The authors would like to acknowledge the outstanding contributions of the staff and trainees of their respective laboratories to the development of liposome technologies. We also thank the following agencies and companies for their funding support: Canadian Institutes for Health Research, Canadian Breast Cancer Association, National Cancer Institute of Canada, National Science and Engineering Research Council of Canada, Canada Foundation for Innovation, Centre for Drug Research and Development, Alza Pharmaceuticals, Tekmira Pharmaceuticals, Alnylam Pharmaceuticals, AlCana Technologies, and Precision NanoSystems.

References

- [1] A.G. Allison, G. Gregoriadis, Liposomes as immunological adjuvants, *Nature* 252 (1974) 252.
- [2] D.S. Watson, A.N. Endsley, L. Huang, Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens, *Vaccine* 30 (2012) 2256–2272.
- [3] B.A. Goins, W.T. Phillips, The use of scintigraphic imaging as a tool in the development of liposomal formulations, *Prog. Lipid Res.* 40 (2001) 95–123.
- [4] M. Dunne, J. Zheng, J. Rosenblat, D.A. Jaffray, C. Allen, APN/CD13-targeting as a strategy to alter the tumor accumulation of liposomes, *J. Control. Release* 154 (2011) 298–305.
- [5] J.A. Bouwstra, P.L. Honeywell-Nguyen, Skin structure and mode of action of vesicles, *Adv. Drug Deliv. Rev.* 54 (Suppl. 1) (2002) S41–S55.
- [6] Y. Rahimpour, H. Hamishehkar, Liposomes in cosmeceuticals, *Expert Opin. Drug Deliv.* 9 (2012) 443–455.
- [7] V.P. Torchilin, Recent approaches to intracellular delivery of drugs and DNA and organelle targeting, *Annu. Rev. Biomed. Eng.* 8 (2006) 343–375.
- [8] V. Weissig, From serendipity to mitochondria-targeted nanocarriers, *Pharm. Res.* 28 (2011) 2657–2668.
- [9] S. Muro, M. Kovai, V. Muzykantov, Endothelial endocytic pathways: gates for vascular drug delivery, *Curr. Vasc. Pharmacol.* 2 (2004) 281–299.
- [10] A.D. Bangham, M.M. Standish, J.C. Watkins, Diffusion of univalent ions across the lamellae of swollen phospholipids, *J. Mol. Biol.* 13 (1965) 238–252.
- [11] A.D. Bangham, M.M. Standish, J.C. Watkins, G. Weissmann, The diffusion of ions from a phospholipid model membrane system, *Protoplasma* 63 (1967) 183–187.
- [12] D. Papahadjopoulos, J.C. Watkins, Phospholipid model membranes. II. Permeability properties of hydrated liquid crystals, *Biochim. Biophys. Acta* 135 (1967) 639–652.
- [13] D.W. Deamer, From "banghasomes" to liposomes: a memoir of Alec Bangham, 1921–2010, *FASEB J.* 24 (2010) 1308–1310.
- [14] S. Batzri, E.D. Korn, Single bilayer liposomes prepared without sonication, *Biochim. Biophys. Acta* 16 (1973) 1015–1019.
- [15] G. Gregoriadis, B.E. Ryman, Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases, *Biochem. J.* 124 (1971) 58P.
- [16] G. Gregoriadis, Drug entrapment in liposomes, *FEBS Lett.* 36 (1973) 292–296.
- [17] G. Gregoriadis, The carrier potential of liposomes in biology and medicine. Part 1, *N. Engl. J. Med.* 295 (1976) 704–710.
- [18] G. Gregoriadis, The carrier potential of liposomes in biology and medicine. Part 2, *N. Engl. J. Med.* 295 (1976) 765–770.
- [19] S.K. Alahari, R. DeLong, M.H. Fisher, N.M. Dean, P. Viliet, R.L. Juliano, Novel chemically modified oligonucleotides provide potent inhibition of P-glycoprotein expression, *J. Pharmacol. Exp. Ther.* 286 (1998) 419–428.
- [20] H.K. Kimelberg, T.F. Tracy, S.M. Biddlecome, R.S. Bourke, The effect of entrapment in liposomes on the in vivo distribution of ³H-methotrexate in a primate, *Cancer Res.* 36 (1976) 2949–2957.

[21] G. Poste, D. Papahadjopoulos, Lipid vesicles as carriers for introducing materials into cultured cells: influence of vesicle lipid composition on mechanism(s) of vesicle incorporation into cells, *Proc. Natl. Acad. Sci. U.S.A.* 73 (1976) 1603–1607.

[22] R.L. Juliano, D. Stamp, Pharmacokinetics of liposome-entrapped anti-tumor drugs, *Biochem. Pharmacol.* 27 (1978) 21–27.

[23] F. Szoka, D. Papahadjopoulos, Procedure for preparation of liposomes with large internal aqueous space and high capture by reverse-phase evaporation, *Proc. Natl. Acad. Sci. U.S.A.* 75 (1978) 4194–4198.

[24] F. Olson, C.A. Hunt, F.C. Szoka, W.J. Vail, D. Papahadjopoulos, Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes, *Biochim. Biophys. Acta* 557 (1979) 9–23.

[25] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Production of large unilamellar vesicles by a rapid extrusion procedure. Characterization of size distribution, trapped volume and ability to maintain a membrane potential, *Biochim. Biophys. Acta* 812 (1985) 55–65.

[26] L.D. Mayer, M.J. Hope, P.R. Cullis, Vesicles of variable sizes produced by a rapid extrusion procedure, *Biochim. Biophys. Acta* 858 (1986) 161–168.

[27] C.H. Huang, Studies on phosphatidylcholine vesicles: formation and physical characteristics, *Biochemistry* 8 (1969) 344–352.

[28] I.V. Zhigaltsev, N. Belliveau, I. Hafez, A.K. Leung, J. Huft, C. Hansen, P.R. Cullis, Bottom-up design and synthesis of limit size lipid nanoparticle systems with aqueous and triglyceride cores using millisecond microfluidic mixing, *Langmuir* 28 (2012) 3633–3640.

[29] T. Kobayashi, S. Tsukagoshi, Y. Sakurai, Enhancement of the cancer chemotherapeutic effect of cytosine arabinoside entrapped in liposomes on mouse leukemia L-1210, *Gann* 66 (1975) 719–720.

[30] E. Mayhew, D. Papahadjopoulos, Y.M. Rustum, C. Dave, Inhibition of tumour cell growth in vitro and in vivo by 1- β -D-arabinofuranosylcytosine entrapped within in phospholipid vesicles, *Cancer Res.* 36 (1976) 4406–4411.

[31] C.R. Alving, E.A. Steck, W.L. Chapman Jr., V.B. Waits, L.D. Hendricks, G.M. Swartz Jr., W.L. Hanson, Therapy of leishmaniasis: superior efficacies of liposome-encapsulated drugs, *Proc. Natl. Acad. Sci. U.S.A.* 75 (1978) 2959–2963.

[32] A. Kedar, E. Mayhew, R.H. Moore, P. Williams, G.P. Murphy, Effect of actinomycin D-containing lipid vesicles on murine renal adenocarcinoma, *J. Surg. Oncol.* 15 (1980) 363–365.

[33] L.G. Cleland, B.V. Roberts, R. Garrett, T.M. Allen, Cortisol palmitate liposomes: enhanced anti-inflammatory effect in rats compared with free cortisol, *Agents Actions* 12 (1982) 348–352.

[34] R.T. Mehta, G. Lopez-Berestein, R. Hopfer, K. Mills, R.T. Juliano, Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells, *Biochim. Biophys. Acta* 770 (1984) 230–234.

[35] A. Gabizon, A. Dagan, D. Goren, Y. Barenholz, Z. Fuks, Liposomes as in vivo carriers of adriamycin: reduced cardiac uptake and preserved antitumor activity in mice, *Cancer Res.* 42 (1982) 4734–4739.

[36] G. Lopez-Berestein, V. Fainstein, R. Hopfer, K. Mehta, M.P. Sullivan, M. Keating, M.G. Rosenblum, R. Mehta, M. Luna, E.M. Hersh, J. Reuben, R.L. Juliano, G.P. Bodey, Liposomal amphotericin B for the treatment of systemic fungal infections in patients with cancer: a preliminary study, *J. Infect. Dis.* 151 (1985) 704–710.

[37] A. Gabizon, T. Peretz, A. Sulkes, S. Amselem, R. Ben-Yosef, N. Ben-Baruch, R. Catane, S. Biran, Y. Barenholz, Systemic administration of doxorubicin-containing liposomes in cancer patients: a phase I study, *Eur. J. Cancer Clin. Oncol.* 25 (1989) 1795–1803.

[38] E. Mayhew, D. Papahadjopoulos, Y.M. Rustum, C. Dave, Use of liposomes for the enhancement of the cytotoxic effects of cytosine arabinoside, *Ann. N. Y. Acad. Sci.* 308 (1978) 371–386.

[39] G. Scherphof, F. Roerdink, M. Waite, J. Parks, Disintegration of phosphatidylcholine liposomes in plasma as a result of interaction with high-density lipoproteins, *Biochim. Biophys. Acta* 542 (1978) 296–307.

[40] T.M. Allen, L.G. Cleland, Serum-induced leakage of liposome contents, *Biochim. Biophys. Acta* 597 (1980) 418–426.

[41] J. Senior, G. Gregoriadis, Is half-life of circulating liposomes determined by changes in their permeability? *FEBS Lett.* 145 (1982) 109–114.

[42] P.R. Cullis, Lateral diffusion rates of phosphatidylcholine in vesicle membranes: effects of cholesterol and hydrocarbon phase transitions, *FEBS Lett.* 70 (1976) 223–228.

[43] T.J. McIntosh, The effect of cholesterol on the structure of phosphatidylcholine bilayers, *Biochim. Biophys. Acta* 513 (1978) 43–58.

[44] G. Storm, F.H. Roerdink, P.A. Steerenberg, W.H. de Jong, D.J. Crommelin, Influence of lipid composition on the antitumor activity exerted by doxorubicin-containing liposomes in a rat solid tumor model, *Cancer Res.* 47 (1987) 3366–3372.

[45] P.R. Cullis, M.J. Hope, The bilayer stabilizing role of sphingomyelin in the presence of cholesterol: a 31P NMR study, *Biochim. Biophys. Acta* 597 (1980) 533–542.

[46] T.M. Allen, A study of phospholipid interactions between high-density lipoproteins and small unilamellar vesicles, *Biochim. Biophys. Acta* 640 (1981) 385–397.

[47] M.H. Bartoli, M. Boitard, H. Fessi, H. Beriel, J.P. Devissaguet, F. Picot, F. Puisieux, In vitro and in vivo antitumoral activity of free, and encapsulated taxol, *J. Microencapsul.* 7 (1990) 191–197.

[48] A. Cabanes, K.E. Briggs, P.C. Gokhale, J.A. Treat, A. Rahman, Comparative in vivo studies with paclitaxel and liposome-encapsulated paclitaxel, *Int. J. Oncol.* 12 (1998) 1035–1040.

[49] E.M. Bolotin, R. Cohen, L.K. Bar, S.N. Emanuel, D.D. Lasic, Y. Barenholz, Ammonium sulphate gradients for efficient and stable remote loading of amphiphatic weak bases into liposomes and ligandosomes, *J. Liposome Res.* 4 (1994) 455–479.

[50] L.D. Mayer, L.C. Tai, M.B. Bally, G.N. Mitilenes, R.S. Ginsberg, P.R. Cullis, Characterization of liposomal systems containing doxorubicin entrapped in response to pH gradients, *Biochim. Biophys. Acta* 1025 (1990) 143–151.

[51] D.C. Drummond, C.O. Noble, Z. Guo, K. Hong, J.W. Park, D.B. Kirpotin, Development of a highly active nanoliposomal irinotecan using a novel intraliposomal stabilization strategy, *Cancer Res.* 66 (2006) 3271–3277.

[52] D.W. Deamer, R.C. Prince, A.R. Crofts, The response of fluorescent amines to pH gradients across liposome membranes, *Biochim. Biophys. Acta* 274 (1972) 323–335.

[53] L.D. Mayer, M.B. Bally, P.R. Cullis, Uptake of adriamycin into large unilamellar vesicles in response to a pH gradient, *Biochim. Biophys. Acta* 857 (1986) 123–126.

[54] T.D. Madden, P.R. Harrigan, L.C. Tai, M.B. Bally, L.D. Mayer, T.E. Redelmeier, H.C. Loughrey, C.P. Tilcock, L.W. Reinish, P.R. Cullis, The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient: a survey, *Chem. Phys. Lipids* 53 (1990) 37–46.

[55] N. Maurer, K.F. Wong, M.J. Hope, P.R. Cullis, Anomalous solubility behavior of the antibiotic ciprofloxacin encapsulated in liposomes: a 1H-NMR study, *Biochim. Biophys. Acta* 1374 (1998) 9–20.

[56] M.J. Johnston, K. Edwards, G. Karlsson, P.R. Cullis, Influence of drug-to-lipid ratio on drug release properties and liposome integrity in liposomal doxorubicin formulations, *J. Liposome Res.* 18 (2008) 145–157.

[57] I.V. Zhigaltsev, G. Winters, M. Srinivasulu, J. Crawford, M. Wong, L. Amankwa, D. Waterhouse, D. Masin, M. Webb, N. Harasym, L. Heller, M.B. Bally, M.A. Ciufolini, P.R. Cullis, N. Maurer, Development of a weak-base docetaxel derivative that can be loaded into lipid nanoparticles, *J. Control. Release* 144 (2010) 332–340.

[58] K.M. Laginha, S. Verwoert, G.J. Charrois, T.M. Allen, Determination of doxorubicin levels in whole tumor and tumor nuclei in murine breast cancer tumors, *Clin. Cancer Res.* 11 (2005) 6944–6949.

[59] M.J. Johnston, S.C. Semple, S.K. Klimuk, K. Edwards, M.L. Eisenhardt, E.C. Leng, G. Karlsson, D. Yanko, P.R. Cullis, Therapeutically optimized rates of drug release can be achieved by varying the drug-to-lipid ratio in liposomal vincristine formulations, *Biochim. Biophys. Acta* 1758 (2006) 55–64.

[60] N.L. Boman, L.D. Mayer, P.R. Cullis, Optimization of the retention properties of vincristine in liposomal systems, *Biochim. Biophys. Acta* 1152 (1993) 253–258.

[61] N.L. Boman, D. Masin, L.D. Mayer, P.R. Cullis, M.B. Bally, Liposomal vincristine which exhibits increased drug retention and increased circulation longevity cures mice bearing P388 tumors, *Cancer Res.* 54 (1994) 2830–2833.

[62] G.J.R. Charrois, T.M. Allen, Drug release rate influences the pharmacokinetics, biodistribution, therapeutic activity, and toxicity of pegylated liposomal doxorubicin formulations in murine breast cancer, *Biochim. Biophys. Acta* 1663 (2004) 167–177.

[63] G. Gregoriadis, D. Neerunjun, Control of the rate of hepatic uptake and catabolism of liposome-entrapped proteins injected into rats. Possible therapeutic applications, *Eur. J. Biochem.* 47 (1974) 179–185.

[64] R.L. Juliano, D. Stamp, The effect of particle size and charge on the clearance rates of liposomes and liposome encapsulated drugs, *Biochim. Biophys. Res. Commun.* 63 (1975) 651–658.

[65] H. Ellens, E. Mayhew, Y.M. Rustum, Reversible depression of the reticuloendothelial system by liposomes, *Biochim. Biophys. Acta* 714 (1982) 479–485.

[66] M.J. Hsu, R.L. Juliano, Interactions of liposomes with the reticuloendothelial system. Nonspecific and receptor-mediated uptake of liposomes by mouse peritoneal macrophages, *Biochim. Biophys. Acta* 720 (1982) 411–419.

[67] T.M. Allen, L. Murray, S. MacKeigan, M. Shah, Chronic liposome administration in mice: effects on reticuloendothelial function and tissue distribution, *J. Pharmacol. Exp. Ther.* 229 (1984) 267–275.

[68] R.M. Abra, M.E. Bosworth, C.A. Hunt, Liposome disposition in vivo: effects of pre-dosing with liposomes, *Res. Commun. Chem. Pathol. Pharmacol.* 29 (1980) 349–360.

[69] Y.J. Kao, R.L. Juliano, Interactions of liposomes with the reticuloendothelial system. Effects of reticuloendothelial blockade on the clearance of large unilamellar vesicles, *Biochim. Biophys. Acta* 677 (1981) 453–461.

[70] R.T. Proffitt, L.E. Williams, C.A. Presant, G.W. Tin, J.A. Uliana, R.C. Gamble, J.D. Baldeschwieler, Liposomal blockade of the reticuloendothelial system: improved tumor imaging with small unilamellar vesicles, *Science* 220 (1983) 502–505.

[71] K.J. Hwang, M.M. Padki, D.D. Chow, H.E. Essien, J.Y. Lai, P.L. Beaumier, Uptake of small liposomes by non-reticuloendothelial tissues, *Biochim. Biophys. Acta* 901 (1987) 88–96.

[72] D. Hoekstra, G. Scherphof, Effect of fetal calf serum and serum protein fractions on the uptake of liposomal phosphatidylcholine by rat hepatocytes in primary monolayer culture, *Biochim. Biophys. Acta* 551 (1979) 109–121.

[73] S.M. Moghim, H.M. Patel, Differential properties of organ-specific serum opsonins for liver and spleen macrophages, *Biochim. Biophys. Acta* 984 (1989) 379–383.

[74] A. Chonn, S.C. Semple, P.R. Cullis, Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes, *J. Biol. Chem.* 267 (1992) 18759–18765.

[75] T.M. Allen, A. Chonn, Large unilamellar liposomes with low uptake into the reticuloendothelial system, *FEBS Lett.* 223 (1987) 42–46.

[76] T.M. Allen, Stealth liposomes: avoiding reticuloendothelial uptake, in: G. Lopez-Berestein, I. Fidler (Eds.), *Liposomes in the Therapy of Infectious Disease and Cancer, UCLA Symposium in Molecular and Cellular Biology* CRC Press, Boca Raton, FL, 1989, pp. 405–415.

[77] A. Abuchowski, J.R. McCoy, N.C. Palczuk, T. Van Es, F.F. Davis, Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase, *J. Biol. Chem.* 252 (1977) 3582–3586.

[78] A.L. Klibanov, K. Maruyama, V.P. Torchilin, L. Huang, Amphiphatic polyethyleneglycols effectively prolong the circulation time of liposomes, *FEBS Lett.* 268 (1990) 235–237.

[79] G. Blume, G. Cevc, Liposomes for the sustained drug release in vivo, *Biochim. Biophys. Acta* 1029 (1990) 91–97.

[80] T.M. Allen, C.B. Hansen, F. Martin, C. Redemann, A. Yau-Young, Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo, *Biochim. Biophys. Acta* 1066 (1991) 29–36.

[81] K. Maruyama, T. Yuda, A. Okamoto, C. Ishikura, S. Kojima, M. Iwatsuru, Effect of molecular weight in amphiphatic polyethyleneglycol on prolonging the circulation time of large unilamellar liposomes, *Chem. Pharm. Bull.(Tokyo)* 39 (1991) 1620–1622.

[82] J. Senior, C. Delgado, D. Fisher, C. Tilcock, G. Gregoriadis, Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies with the poly(ethylene glycol)-coated vesicles, *Biochim. Biophys. Acta* 1062 (1991) 77–82.

[83] D. Papahadjopoulos, T.M. Allen, A. Gabizon, E. Mayhew, K. Matthay, S.K. Huang, K.D. Lee, M.C. Woodle, D.D. Lasic, C. Redemann, F.J. Martin, Sterically stabilized liposomes: improvements in pharmacokinetics and antitumor therapeutic efficacy, *Proc. Natl. Acad. Sci. U. S. A.* 88 (1991) 11460–11464.

[84] T.M. Allen, C.B. Hansen, Pharmacokinetics of Stealth versus conventional liposomes: effect of dose, *Biochim. Biophys. Acta* 1068 (1991) 133–141.

[85] T. Ishida, R. Maeda, M. Ichihara, K. Irimura, H. Kiwada, Accelerated clearance of PEGylated liposomes in rats after repeated injections, *J. Control. Release* 88 (2003) 35–42.

[86] T.M. Allen, T. Mehra, C.B. Hansen, Y.C. Chin, Stealth liposomes: an improved sustained release system for 1-b-D-arabinofuranosylcytosine, *Cancer Res.* 52 (1992) 2431–2439.

[87] E.G. Mayhew, D. Lasic, S. Babbar, F.J. Martin, Pharmacokinetics and antitumor activity of epirubicin encapsulated in long-circulating liposomes incorporating a polyethylene glycol-derivatized phospholipid, *Int. J. Cancer* 51 (1992) 302–309.

[88] J. Vaage, E. Mayhew, D. Lasic, F. Martin, Therapy of primary and metastatic mouse mammary carcinomas with doxorubicin encapsulated in long circulating liposomes, *Int. J. Cancer* 51 (1992) 942–948.

[89] I.A.J.M. Bakker-Woudenberg, A.F. Lokerse, M.T. ten Kate, G. Storm, Enhanced localization of liposomes with prolonged blood circulation time in infected lung tissue, *Biochim. Biophys. Acta* 1138 (1992) 318–326.

[90] M.C. Woodle, T.M. Allen, E. Mayhew, P.S. Uster, In vivo efficacy of vincristine entrapped in long-circulating liposomes, *Proc. Am. Assoc. Cancer Res.* 33 (1992) 447.

[91] M.C. Woodle, G. Storm, M.S. Newman, J.J. Jekot, L.R. Collins, F.J. Martin, F.C. Szoka, Prolonged systemic delivery of peptide drugs by long-circulating liposomes: illustration with vasopressin in the Brattleboro rat, *Pharm. Res.* 9 (1992) 260–265.

[92] A. Gabizon, R. Catane, B. Uziely, B. Kaufman, T. Safra, R. Cohen, F. Martin, A. Huang, Y. Barenholz, Prolonged circulation time and enhanced accumulation in malignant exudates of doxorubicin encapsulated in polyethylene-glycol coated liposomes, *Cancer Res.* 54 (1994) 987–992.

[93] N.D. James, R.J. Coker, D. Tomlinson, J.R. Harris, M. Gompels, A.J. Pinching, J.S. Stewart, Liposomal doxorubicin (Doxil): an effective new treatment for Kaposi's sarcoma in AIDS, *Clin. Oncol. (R. Coll. Radiol.)* 6 (1994) 294–296.

[94] C. Kelly, C. Jefferies, S.A. Cryan, Targeted liposomal drug delivery to monocytes and macrophages, *J. Drug Deliv.* 2011 (2011) 727241.

[95] G. Weissmann, C. Cohen, S. Hoffstein, Introduction of missing enzymes into the cytoplasm of cultured mammalian cells by means of fusion-prone liposomes, *Trans. Assoc. Am. Physicians* 89 (1976) 171–183.

[96] M. Ozawa, A. Asano, The preparation of cell fusion-inducing proteoliposomes from purified glycoproteins of HVJ (Sendai virus) and chemically defined lipids, *J. Biol. Chem.* 256 (1981) 5954–5956.

[97] R.A. Parente, S. Nir, F.C.J. Szoka, pH-dependent fusion of phosphatidylcholine small vesicles. Induction by a synthetic amphiphatic peptide, *J. Biol. Chem.* 263 (1988) 4724–4730.

[98] A. Bailey, M.A. Monck, P.R. Cullis, pH-induced destabilization of lipid bilayers by a lipopeptide derived from influenza hemagglutinin, *Biochim. Biophys. Acta* 1324 (1997) 232–244.

[99] V.P. Torchilin, R. Rammohan, V. Weissig, T.S. Levchenko, TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors, *Proc. Natl. Acad. Sci. U.S.A.* 98 (2001) 8786–8791.

[100] J. Weinstein, R. Blumenthal, S. Sharow, P. Henkert, Antibody-mediated targeting of liposomes. Binding to lymphocytes does not ensure incorporation of vesicle contents into the cells, *Biochim. Biophys. Acta* 509 (1978) 272–288.

[101] L.D. Leserman, J. Barbet, F. Kourilsky, J.N. Weinstein, Targeting to cells of fluorescent liposomes covalently coupled with monoclonal antibody or protein A, *Nature* 288 (1980) 602–604.

[102] L.D. Leserman, J.N. Weinstein, R. Blumenthal, W.D. Terry, Receptor-mediated endocytosis of antibody-opsonized liposomes by tumor cells, *Proc. Natl. Acad. Sci. U. S. A.* 77 (1980) 4089–4093.

[103] R.M. Straubinger, K. Hong, D.S. Friend, D. Papahadjopoulos, Endocytosis of liposomes and intracellular fate of encapsulated molecules: encounter with a low pH compartment after internalization in coated vesicles, *Cell* 32 (1983) 1069–1079.

[104] C.P. Leamon, P.S. Low, Delivery of macromolecules into living cells: a method that exploits folate receptor endocytosis, *Proc. Natl. Acad. Sci. U.S.A.* 88 (1991) 5572–5576.

[105] T.D. Heath, R.T. Fraley, D. Papahdjopoulos, Antibody targeting of liposomes: cell specificity obtained by conjugation of F(ab')₂ to vesicle surface, *Science* 210 (1980) 539–541.

[106] F.J. Martin, W.I. Hubbell, D. Papahadjopoulos, Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds, *Biochemistry* 20 (1981) 4229–4238.

[107] T.D. Heath, J.A. Montgomery, J.R. Piper, D. Papahadjopoulos, Antibody-targeted liposomes: increase in specific toxicity of methotrexate-gamma-aspartate, *Proc. Natl. Acad. Sci. U.S.A.* 80 (1983) 1377–1381.

[108] D. Papahadjopoulos, A. Gabizon, Targeting of liposomes to tumor cells in vivo, *Ann. N. Y. Acad. Sci.* 507 (1987) 64–74.

[109] K. Maruyama, E. Holmber, S.J. Kennel, A. Klibanov, V. Torchilin, L. Huang, Characterization of in vivo immunoliposome targeting to pulmonary endothelium, *J. Pharm. Sci.* 79 (1990) 978–984.

[110] A.L. Klibanov, K. Maruyama, A.M. Beckerleg, V.P. Torchilin, L. Huang, Activity of amphiphatic poly(ethyleneglycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target, *Biochim. Biophys. Acta* 1062 (1991) 142–148.

[111] A. Mori, A.L. Klibanov, V.P. Torchilin, L. Huang, Influence of the steric barrier of amphiphatic poly(ethyleneglycol) and ganglioside GM₁ on the circulation time of liposomes and on the target binding of immunoliposomes *in vivo*, *FEBS Lett.* 284 (1991) 263–266.

[112] K. Maruyama, T. Takizawa, T. Yuda, S.J. Kennel, L. Huang, M. Iwatsuru, Targetability of novel immunoliposomes modified with amphiphatic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies, *Biochim. Biophys. Acta* 1234 (1995) 74–80.

[113] V.P. Torchilin, A.L. Klibanov, L. Huang, S. O'Donnell, N.D. Nossiff, B.A. Khaw, Targeted accumulation of polyethylene glycol-coated immunoliposomes in infarcted rabbit myocardium, *FASEB J.* 6 (1992) 2716–2719.

[114] I. Ahmad, T.M. Allen, Antibody-mediated specific binding and cytotoxicity of liposome-entrapped doxorubicin to lung cancer cells *in vitro*, *Cancer Res.* 52 (1992) 4817–4820.

[115] G. Blume, G. Cevc, M.D. Crommelin, L.A. Bakker-Woudenberg, C. Kluft, G. Storm, Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times, *Biochim. Biophys. Acta* 1149 (1993) 180–184.

[116] T.M. Allen, A.K. Agrawal, I. Ahmad, C.B. Hansen, S. Zalipsky, Antibody-mediated targeting of long-circulating (Stealth®) liposomes, *J. Liposome Res.* 4 (1994) 1–25.

[117] R.J. Lee, P.S. Low, Delivery of liposomes into cultured KB cells via folate receptor-mediated endocytosis, *J. Biol. Chem.* 269 (1994) 3198–3204.

[118] C.B. Hansen, G.Y. Kao, E.H. Moase, S. Zalipsky, T.M. Allen, Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures, *Biochim. Biophys. Acta* 1239 (1995) 133–144.

[119] T.M. Allen, E. Brandeis, C.B. Hansen, G.Y. Kao, S. Zalipsky, A new strategy for attachment of antibodies to sterically stabilized liposomes resulting in efficient targeting to cancer cells, *Biochim. Biophys. Acta* 1237 (1995) 99–108.

[120] S. Suzuki, S. Watanabe, T. Masuko, Y. Hashimoto, Preparation of long-circulating immunoliposomes containing adriamycin by a novel method to coat immunoliposomes with poly(ethylene glycol), *Biochim. Biophys. Acta* 124 (1995) 9–16.

[121] I. Ahmad, M. Longenecker, J. Samuel, T.M. Allen, Antibody-targeted delivery of doxorubicin entrapped in sterically stabilized liposomes can eradicate lung cancer in mice, *Cancer Res.* 53 (1993) 1484–1488.

[122] T. Ishida, D.L. Iden, T.M. Allen, A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs, *FEBS Lett.* 460 (1999) 129–133.

[123] D.L. Iden, T.M. Allen, In vitro and *in vivo* comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion technique, *Biochim. Biophys. Acta* 1513 (2001) 207–216.

[124] J.W. Park, D.B. Kirpotin, K. Hong, R. Shalaby, Y. Shao, U.B. Nielsen, J.D. Marks, D. Papahadjopoulos, C.C. Benz, Tumor targeting using anti-her2 immuno-liposomes, *J. Control. Release* 74 (2001) 95–113.

[125] D.E. Lopes de Menezes, L.M. Pilarski, T.M. Allen, In vitro and *in vivo* targeting of immunoliposomal doxorubicin to human B-cell lymphoma, *Cancer Res.* 58 (1998) 3320–3330.

[126] J.W. Park, K. Hong, D.B. Kirpotin, G. Colbern, R. Shalaby, J. Baselga, Y. Shao, U.B. Nielsen, J.D. Marks, D. Moore, D. Papahadjopoulos, C.C. Benz, Anti-HER2 immunoliposomes: enhanced efficacy attributable to targeted delivery, *Clin. Cancer Res.* 8 (2002) 1172–1181.

[127] M.H. Vingerhoeds, P.A. Steerenberg, J.J. Hendriks, L.C. Dekker, Q.G. Van Hoesel, D.J. Crommelin, G. Storm, Immunoliposome-mediated targeting of doxorubicin to human ovarian carcinoma *in vitro* and *in vivo*, *Br. J. Cancer* 74 (1996) 1023–1029.

[128] D. Goren, A.T. Horowitz, S. Zalipsky, M.C. Woodle, Y. Yarden, A. Gabizon, Targeting of stealth liposomes to erb-2 (Her2) receptor: *in vitro* and *in vivo* studies, *Br. J. Cancer* 74 (1996) 1749–1756.

[129] D.B. Kirpotin, D.C. Drummond, Y. Shao, M.R. Shalaby, K. Hong, U.B. Nielsen, J.D. Marks, C.C. Benz, J.W. Park, Antibody targeting of long-circulating lipidic nanoparticles does not increase tumor localization but does increase internalization in animal models, *Cancer Res.* 66 (2006) 6732–6740.

[130] K. Riviere, Z. Huang, K. Jerger, N. Macaraeg, F.C.J. Szoka, Antitumor effect of folate-targeted liposomal doxorubicin in KB tumor-bearing mice after intravenous administration, *J. Drug Target.* 19 (2011) 14–24.

[131] T.M. Allen, Ligand-targeted therapeutics in anticancer therapy, *Nat. Rev. Cancer* 2 (2002) 750–763.

[132] P. Sapra, P. Tyagi, T.M. Allen, Ligand-targeted liposomes for cancer treatment, *Curr. Drug Deliv.* 2 (2005) 369–381.

[133] T. Lammers, F. Kiessling, W.E. Hennink, G. Storm, Drug targeting to tumors: principles, pitfalls and (pre-) clinical progress, *J. Control. Release* 161 (2) (2012) 175–187.

[134] R.I. Jølck, L.N. Feldborg, S. Andersen, S.M. Moghimi, T.L. Andrensen, Engineering liposomes and nanoparticles for biological targeting, *Adv. Biochem. Eng. Biotechnol.* 125 (2011) 251–280.

[135] R.R. Sawant, V.P. Torchilin, Challenges in development of targeted liposomal therapeutics, *AAPS J.* 14 (2) (2012) 303–315.

[136] R.K. Jain, T. Stylianopoulos, Delivering nanomedicine to solid tumors, *Nat. Rev. Clin. Oncol.* 7 (2010) 653–664.

[137] G.J.R. Charrois, T.M. Allen, Rate of biodistribution of STEALTH® liposomes to tumor and skin: influence of liposome diameter and implications for toxicity and therapeutic activity, *Biochim. Biophys. Acta* 1609 (2003) 102–108.

[138] M.R. Dreher, W. Liu, C.R. Michelich, M.W. Dewhirst, F. Yuan, A. Chilkoti, Tumor vascular permeability, accumulation, and penetration of macromolecular drug carriers, *J. Natl. Cancer Inst.* 98 (2006) 335–344.

[139] Z. Popović, W. Liu, V.P. Chauhan, J. Lee, C. Wong, A.B. Greytak, N. Insin, D.G. Nocera, D. Fukumura, J.R. RK., M.G. Bawendi, A nanoparticle size series for in vivo fluorescence imaging, *Angew. Chem. Int. Ed Engl.* 49 (2010) 8649–8652.

[140] K.D. Orcutt, J.J. Rhoden, B. Ruiz-Yi, J.V. Frangioni, K.D. Wittrup, Effect of small molecule binding affinity on tumor uptake in vivo, *Mol. Cancer Ther.* 11 (6) (2012) 1365–1372.

[141] M. Juweid, R. Neumann, C. Paik, M.J. Perez-Bacete, J. Sato, W. van Osdol, J.N. Weinstein, Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier, *Cancer Res.* 52 (1992) 5144–5153.

[142] T.D. Heath, R.T. Fraley, J. Bentz, E.W.J. Voss, J.N. Herron, D. Papahadjopoulos, Antibody-directed liposomes. Determination of affinity constants for soluble and liposome-bound antifluorescein, *Biochim. Biophys. Acta* 770 (1984) 148–158.

[143] J.N. Weinstein, W. van Osdol, Early intervention in cancer using monoclonal antibodies and other biological ligands: micropharmacology and the “binding site barrier”, *Cancer Res.* 52 (1992) 2747–2751.

[144] F. Pastorino, C. Brignole, D. Marimpietri, M. Cilli, C. Gambini, D. Ribatti, R. Longhi, T.M. Allen, A. Corti, M. Ponzone, Vascular damage and anti-angiogenic effects of tumor vessel-targeted liposomal chemotherapy, *Cancer Res.* 63 (2003) 7400–7409.

[145] E. Moase, W. Qi, T. Ishida, Z. Gabos, B.M. Longenecker, G.L. Zimmermann, L. Ding, M. Krantz, T.M. Allen, Anti-MUC-1 immunoliposomal doxorubicin in the treatment of murine models of metastatic breast cancer, *Biochim. Biophys. Acta* 1510 (2001) 43–55.

[146] P. Sapra, T.M. Allen, Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs, *Cancer Res.* 62 (2002) 7190–7194.

[147] K. Laginha, D. Mumbengegwı, T. Allen, Liposomes targeted via two different antibodies: assay, B-cell binding and cytotoxicity, *Biochim. Biophys. Acta* 1711 (2005) 25–32.

[148] R. Suzuki, T. Takizawa, Y. Kuwata, M. Mutoh, N. Ishiguro, N. Utoguchi, A. Shinohara, M. Eriguchi, H. Yanagie, K. Maruyama, Effective anti-tumor activity of oxaliplatin encapsulated in transferrin-PEG-liposome, *Int. J. Pharm.* 346 (2008) 143–150.

[149] D.F. Nellis, S.L. Giardina, G.M. Janini, S.R. Shenoy, J.D. Marks, R. Tsai, D.C. Drummond, K. Hong, J.W. Park, T.F. Ouellette, S.C. Perkins, D.B. Kirpotin, Preclinical manufacture of anti-HER2 liposome-inserting, scFv-PEG-lipid conjugate. 2. Conjugate micelle identity, purity, stability, and potency analysis, *Biotechnol. Prog.* 21 (2005) 221–232.

[150] L. Xu, W.H. Tang, C.C. Huang, W. Alexander, L.M. Xiang, K.F. Pirolo, A. Rait, E.H. Chang, Systemic p53 gene therapy of cancer with immunolipoplexes targeted by anti-transferrin receptor scFv, *Mol. Med.* 7 (2001) 723–734.

[151] S. Bibi, E. Lattmann, A.R. Mohammed, Y. Perrie, Trigger release liposome systems: local and remote controlled delivery? *J. Microencapsul.* 29 (2012) 262–276.

[152] J.N. Weinstein, R.L. Magin, M.B. Yatvin, D.S. Zaharko, Liposomes and local hyperthermia: selective delivery of methotrexate to heated tumors, *Science* 204 (1979) 188–191.

[153] M.B. Yatvin, W. Kreutz, B.A. Horwitz, M. Shinitzky, pH sensitive liposomes: possible clinical implications, *Science* 210 (1980) 1253–1255.

[154] S.K. Huang, P.R. Stauffer, K. Hong, J.W. Guo, T.L. Phillips, A. Huang, D. Papahadjopoulos, Liposomes and hyperthermia in mice: increased tumor uptake and therapeutic efficacy of doxorubicin in sterically stabilized liposomes, *Cancer Res.* 54 (1994) 2186–2191.

[155] S. Ning, K. Macleod, R.M. Abra, A.H. Huang, G.M. Hahn, Hyperthermia induces doxorubicin release from long-circulating liposomes and enhances their anti-tumor efficacy, *Int. J. Radiat. Oncol. Biol. Phys.* 29 (1994) 827–834.

[156] X. Guo, F.C.J. Szoka, Steric stabilization of fusogenic liposomes by a low-pH sensitive PEG-diorthoester-lipid conjugate, *Bioconjug. Chem.* 12 (2001) 291–300.

[157] T. Ishida, M.J. Kirchmeier, E.H. Moase, S. Zalipsky, T.M. Allen, Targeted delivery and triggered release of liposomal doxorubicin enhances cytotoxicity against human B lymphoma cells, *Biochim. Biophys. Acta* 1515 (2001) 144–158.

[158] S. Simoes, J.N. Moreira, C. Fonseca, N. Duzgunes, M.C. de Lima, On the formulation of pH-sensitive liposomes with long circulation times, *Adv. Drug Deliv. Rev.* 56 (2004) 947–965.

[159] J.X. Zhang, S. Zalipsky, N. Mullah, M. Pechar, T.M. Allen, Pharmacological attributes of dioleoylphosphatidylethanolamine/cholesterylhemisuccinate liposomes containing different types of cleavable lipopolymers, *Pharmacol. Res.* 49 (2004) 185–198.

[160] W. Li, F. Nicol, F.C.J. Szoka, GALA: a designed synthetic pH-responsive amphiphatic peptide with applications in drug and gene delivery, *Adv. Drug Deliv. Rev.* 56 (2004) 967–985.

[161] T.R. Hesketh, S.N. Payne, J.H. Humphrey, Complement and phospholipase C lysis of lipid membrane, *Immunology* 23 (1972) 705–711.

[162] C.K. Kim, K.M. Park, Liposome immunoassay (LIA) for gentamicin using phospholipase C, *J. Immunol. Methods* 170 (1994) 225–231.

[163] J. Davidsen, K. Jørgensen, T.L. Andrensen, O.G. Mouritsen, Secreted phospholipase A(2) as a new enzymatic trigger mechanism for localised liposomal drug release and absorption in diseased tissue, *Biochim. Biophys. Acta* 1609 (2003) 95–101.

[164] C.K. Kim, S.J. Lim, Liposome immunoassay (LIA) with antigen-coupled liposomes containing alkaline phosphatase, *J. Immunol. Methods* 159 (1993) 101–106.

[165] N.R. Sarkar, T. Rosendahl, A.B. Krueger, A.L. Banerjee, K. Benton, S. Mallik, D.K. Srivastava, “Uncorking” of liposomes by matrix metalloproteinase-9, *Chem. Commun. (Camb.)* 8 (2005).

[166] G. Zhu, J.N. Mock, I. Aljuffali, B.S. Cummings, R.D. Arnold, Secretory phospholipase A₂ responsive liposomes, *J. Pharm. Sci.* 100 (2011) 3146–3159.

[167] N. Sarkar, J. Banerjee, A.J. Hanson, A.I. Elegbede, T. Rosendahl, A.B. Krueger, A.L. Banerjee, S. Tobwala, R. Wang, X. Lu, S. Mallik, D.K. Srivastava, Matrix metalloproteinase-assisted triggered release of liposomal contents, *Bioconjg. Chem.* 19 (2008) 57–64.

[168] P.D. Senter, C.J. Springer, Selective activation of anticancer prodrugs by monoclonal antibody-enzyme conjugates, *Adv. Drug Deliv. Rev.* 53 (2001) 247–264.

[169] S.L. Huang, R.C. MacDonald, Acoustically active liposomes for drug encapsulation and ultrasound-triggered release, *Biochim. Biophys. Acta* 1665 (2004) 134–141.

[170] Y. Ueno, S. Sonoda, R. Suzuki, M. Yokouchi, Y. Kawasoe, K. Tachibana, K. Maruyama, T. Sakamoto, S. Komiya, Combination of ultrasound and bubble liposome enhance the effect of doxorubicin and inhibit murine osteosarcoma growth, *Cancer Biol. Ther.* 12 (2011) 270–277.

[171] B. Osiecka, K. Jurczyszyn, K. Symonowicz, A. Bronowicz, P. Ostasiewicz, E. Czapinska, K. Hotowy, M. Krzystek-Korpaczka, E. Gebarowska, I. Izkowska, P. Dziegieł, G. Terlecki, P. Ziolkowski, In vitro and in vivo matrix metalloproteinase expression after photodynamic therapy with a liposomal formulation of aminolevulinic acid and its methyl ester, *Cell. Mol. Biol. Lett.* 15 (2010) 630–650.

[172] A. Yavlovich, A. Singh, R. Blumenthal, A. Puri, A novel class of photo-triggerable liposomes containing DPPC:DC(8,9)PC as vehicles for delivery of doxorubicin to cells, *Biochim. Biophys. Acta* 1808 (2011) 117–126.

[173] K.J. Widder, A.E. Senyei, D.F. Ranney, Magnetically responsive microspheres and other carriers for the biophysical targeting of antitumor agents, *Adv. Pharmacol. Chemother.* 16 (1979) 213–271.

[174] E. Viroonchatapan, H. Sato, M. Ueno, I. Adachi, K. Tazawa, I. Horikoshi, Magnetic targeting of thermosensitive magnetoliposomes to mouse livers in an *in situ* on-line perfusion system, *Life Sci.* 58 (1996) 2251–2261.

[175] W. Su, H. Wang, S. Wang, Z. Liao, S. Kang, Y. Peng, L. Han, J. Chang, PEG/RGD-modified magnetic polymeric liposomes for controlled drug release and tumor cell targeting, *Int. J. Pharm.* 426 (2012) 170–181.

[176] B. Smith, I. Lyakhov, K. Loomis, D. Needie, U. Baxa, A. Yavlovich, J. Capala, R. Blumenthal, A. Puri, Hyperthermia-triggered intracellular delivery of anticancer agent to HER2(+) cells by HER2-specific affibody (ZHER2-GS-Cys)-conjugated thermosensitive liposomes (HER2(+) affisomes), *J. Control. Release* 153 (2011) 187–194.

[177] W. Yang, M. Ahmed, B. Tasawwar, T. Levchenko, R.R. Sawant, M. Collins, S. Signoretto, V. Torchilin, S.N. Goldberg, Radiofrequency ablation combined with liposomal quercetin to increase tumour destruction by modulation of heat shock protein production in a small animal model, *Int. J. Hyperthermia* 27 (2011) 527–538.

[178] M. Ahmed, M. Moussa, S.N. Goldberg, Synergy in cancer treatment between liposomal chemotherapeutics and thermal ablation, *Chem. Phys. Lipids* 165 (2012) 424–437.

[179] R.J. Francis, S.K. Sharma, C. Springer, A.J. Green, L.D. Hope-Stone, L. Sena, J. Martin, K.L. Adamson, A. Robbins, L. Gumbrell, D. O’Malley, E. Tsionpanou, H. Shahbakhti, S. Webley, D. Hochhauser, A.J. Hilson, D. Blakey, R.H. Begent, A phase I trial of antibody directed enzyme prodrug therapy (ADEPT) in patients with advanced colorectal carcinoma or other CEA producing tumours, *Br. J. Cancer* 87 (2002) 600–607.

[180] Z. Vujaskovic, D.W. Kim, E. Jones, L. Lan, L. McCall, M.W. Dewhirst, O. Craciunescu, P. Stauffer, V. Liotcheva, A. Betof, K. Blackwell, A phase I/II study of neoadjuvant liposomal doxorubicin, paclitaxel, and hyperthermia in locally advanced breast cancer, *Int. J. Hyperthermia* 26 (2010) 514–521.

[181] S.K. Sharma, R.B. Pledley, J. Bhatia, G.M. Boxer, E. El-Emir, U. Qureshi, B. Tolner, H. Lowe, N.P. Michael, N. Minton, R.H. Begent, K.A. Chester, Sustained tumor regression of human colorectal cancer xenografts using a multifunctional mannosylated fusion protein in antibody-directed enzyme prodrug therapy, *Clin. Cancer Res.* 11 (2005) 814–825.

[182] A. Mayer, S.K. Sharma, B. Tolner, N.P. Minton, D. Purdy, P. Armitage, G. Tharakorn, R.H. Begent, K.A. Chester, Modifying an immunogenic epitope on a therapeutic protein: a step towards an improved system for antibody-directed enzyme prodrug therapy (ADEPT), *Br. J. Cancer* 90 (2004) 2402–2410.

[183] D. Needham, G. Anyarambhatla, G. Kong, M.W. Dewhirst, A new temperature-sensitive liposome for use with mild hyperthermia: characterization and testing in a human tumour xenograft model, *Cancer Res.* 60 (2000) 1197–1201.

[184] <http://clinicaltrials.gov/ct2/results?term=thermodox>.

[185] R. Fraley, S. Subramani, P. Berg, D. Papahadjopoulos, Introduction of liposome-encapsulated SV40 DNA into cells, *J. Biol. Chem.* 255 (1980) 10431–10435.

[186] R. Fraley, R.M. Straubinger, G. Rule, E.I. Springer, D. Papahadjopoulos, Liposome-mediated delivery of deoxyribonucleic acid to cells: enhanced efficiency of delivery related to lipid composition and incubation conditions, *Biochemistry* 20 (1981) 6978–6987.

[187] R.M. Straubinger, D. Papahadjopoulos, Liposomes as carriers for intracellular delivery of nucleic acids, *Methods Enzymol.* 101 (1983) 512–527.

[188] C. Nicolau, A. Le Pape, P. Soriano, F. Fargette, J.P. Muh, M.F. Juhel, In vivo transfer and expression of the liposome encapsulated rat insulin gene, *Prog. Clin. Biol. Res.* 102 (pt A) (1982) 321–330.

[189] C.M. Rudin, J.L. Marshall, C.H. Huang, H.L. Kindler, C. Zhang, D. Kumar, P.C. Gokhale, J. Steinberg, S. Wanaski, U.N. Kasid, M.J. Ratain, Delivery of a liposomal c-raf-1 antisense oligonucleotide by weekly bolus dosing in patients with advanced solid tumors: a phase I study, *Clin. Cancer Res.* 10 (2004) 7244–7251.

[190] P.L. Felgner, T.R. Gadek, M. Holm, R. Roman, H.W. Chan, M. Wenz, J.P. Northrop, G.M. Ringold, M. Danielsen, Lipofection: a highly efficient, lipid mediated DNA-transfection procedure, *Proc. Natl. Acad. Sci. U. S. A.* 84 (1987) 7413–7417.

[191] J.H. Felgner, R. Kumar, C.N. Sridhar, C.J. Wheeler, Y.J. Tsai, R. Border, P. Ramsey, M. Martin, P.L. Felgner, Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations, *J. Biol. Chem.* 269 (1994) 2550–2561.

[192] H. Mizuguchi, T. Nakagawa, M. Nakanishi, S. Imazu, S. Nakagawa, T. Mayumi, Efficient gene transfer into mammalian cells using fusogenic liposome, *Biochem. Biophys. Res. Commun.* 218 (1996) 402–407.

[193] K.W. Mok, P.R. Cullis, Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA, *Biophys. J.* 73 (1997) 2534–2545.

[194] A. Hirko, F. Tang, J.A. Hughes, Cationic lipid vectors for plasmid DNA delivery, *Curr. Med. Chem.* 10 (2003) 1185–1193.

[195] B. Martin, M. Sainlos, A. Aissaoui, N. Oudrhiri, M. Hauchecorne, J.P. Vigneron, J.M. Lehn, P. Lehn, The design of cationic lipids for gene delivery, *Curr. Pharm. Des.* 11 (2005) 375–394.

[196] K. Lappalainen, I. Jääskeläinen, K. Syrjänen, A. Urtti, S. Syrjänen, Comparison of cell proliferation and toxicity assays using two cationic liposomes, *Pharm. Res.* 11 (1994) 1127–1131.

[197] M.C. Filion, N.C. Phillips, Toxicity and immunomodulatory activity of liposomal vectors formulated with cationic lipids toward immune effector cells, *Biochim. Biophys. Acta* 1329 (1997) 345–356.

[198] J.J. Wheeler, L. Palmer, M. Ossanlou, I. MacLachlan, R.W. Graham, Y.P. Zhang, M.J. Hope, P. Scherrer, P.R. Cullis, Stabilized plasmid-lipid particles: construction and characterization, *Gene Ther.* 6 (1999) 271–281.

[199] D.B. Fenske, I. MacLachlan, P.R. Cullis, Stabilized plasmid-lipid particles: a systemic gene therapy vector, *Methods Enzymol.* 346 (2002) 36–71.

[200] S.C. Semple, S.K. Klimuk, T.O. Harasym, N. Dos Santos, S.M. Ansell, K.F. Wong, N. Maurer, H. Stark, P.R. Cullis, M.J. Hope, P. Scherrer, Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures, *Biochim. Biophys. Acta* 1510 (2001) 152–166.

[201] G. Basha, T.I. Novobrantseva, N. Rosin, Y.Y. Tam, I.M. Hafez, M.K. Wong, T. Sugo, V.M. Ruda, J. Qin, B. Klebanov, M. Ciufolini, A. Akinc, Y.K. Tam, M.J. Hope, P.R. Cullis, Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells, *Mol. Ther.* 19 (2011) 2186–2200.

[202] N. Maurer, K.F. Wong, H. Stark, L. Louie, D. McIntosh, T. Wong, P. Scherrer, S.C. Semple, P.R. Cullis, Spontaneous entrapment of polynucleotides upon electrostatic interaction with ethanol-destabilized cationic liposomes, *Biophys. J.* 80 (2001) 2310–2326.

[203] C. Walker, M. Selby, A. Erickson, D. Cataldo, J.P. Valensi, G.V. Van Nest, Cationic lipids direct a viral glycoprotein into the class I major histocompatibility complex antigen-presentation pathway, *Proc. Natl. Acad. Sci. U. S. A.* 89 (1992) 7915–7918.

[204] B. Mui, S.G. Raney, S.C. Semple, M.J. Hope, Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles, *J. Pharmacol. Exp. Ther.* 298 (2001) 1185–1192.

[205] P. Laing, A. Bacon, B. McCormack, G. Gregoradias, B. Frisch, F. Schuber, The 'co-delivery' approach to liposomal vaccines: application to the development of influenza-A and hepatitis-B vaccine candidates, *J. Liposome Res.* 16 (2006) 229–235.

[206] S. de Jong, G. Chikh, L. Sekirov, S. Raney, S. Semple, S. Klimuk, N. Yuan, M. Hope, P. Cullis, Y. Tam, Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN, *Cancer Immunol. Immunother.* 56 (2007) 1251–1264.

[207] A.D. Judge, G. Bola, A.C. Lee, I. MacLachlan, Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo, *Mol. Ther.* 13 (2006) 494–505.

[208] T.S. Zimmermann, A.C. Lee, A. Akinc, B. Bramlage, D. Bumcrot, M.N. Fedoruk, J. Harborth, J.A. Heyes, L.B. Jeffs, M. John, A.D. Judge, K. Lam, K. McClintock, L.V. Nechev, L.R. Palmer, T. Racie, I. Röhle, S. Seiffert, S. Shanmugam, V. Sood, J. Soutschek, I. Toudjarska, A.J. Wheat, E. Yaworski, W. Zedalis, V. Koteliansky, M. Manoharan, H.P. Vornlocher, I. MacLachlan, RNAi-mediated gene silencing in non-human primates, *Nature* 441 (2006) 111–114.

[209] S.C. Semple, A. Akinc, J. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srivivasulu, M.J. Weinstein, Q. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan, M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, *Nat. Biotechnol.* 28 (2010) 172–176.

[210] A. Akinc, W. Querbes, S. De, J. Qin, M. Frank-Kamenetsky, K.N. Jayaprakash, M. Jayaraman, K.G. Rajeev, W.L. Cantley, J.R. Dorkin, J.S. Butler, L. Qin, T. Racie, A. Sprague, E. Fava, A. Zeigerer, M.J. Hope, M. Zerial, D.W. Sah, K. Fitzgerald, M.A. Tracy, M. Manoharan, V. Koteliansky, A. Fougerolles, M.A. Maier, Targeted delivery of RNAi therapeutics with endogenous and exogenous ligand-based mechanisms, *Mol. Ther.* 18 (2010) 1357–1364.

[211] G.Y. Kao, L.-J. Chang, T.M. Allen, Use of targeted cationic liposomes in enhanced DNA delivery to cancer cells, *Cancer Gene Ther.* 3 (1996) 250–256.

[212] S. Wang, R.J. Lee, G. Cauchon, D.G. Gorenstein, P.S. Low, Delivery of antisense oligodeoxypyrimidine nucleotides against the human epidermal growth factor receptor into cultured KB cells with liposomes conjugated to folate via polyethylene glycol, *Biochemistry* 92 (1995) 3318–3322.

[213] M. Jayaraman, S.M. Ansell, B.L. Mui, Y.K. Tam, J. Chen, X. Du, D. Butler, L. Eltep, S. Matsuda, R. Kallanthottathil, I.M. Hafez, A. Akinc, M.A. Maier, M.A. Tracy, P.R. Cullis, T.D. Madden, M. Manoharan, M.J. Hope, Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo, *Angew. Chem. Int. Ed. Engl.* 51 (34) (2012) 8529–8533.

[214] A. Kawabata, A. Baoum, N. Ohta, S. Jacquez, G.M. Seo, C. Berkland, M. Tamura, Intratracheal administration of a nanoparticle-based therapy with the angiotensin II type 2 receptor gene attenuates lung cancer growth, *Cancer Res.* 72 (8) (2012) 2057–2067.

[215] J. Nguyen, F.C. Szoka, Nucleic acid delivery: the missing pieces of the puzzle, *Acc. Chem. Res.* 45 (7) (2012) 1153–1162.

[216] Alnylam, in.

[217] N. Belliveau, J. Huft, P. Lin, S. Chen, A.K.K. Leung, T.J. Weaver, A.W. Wild, J.B. Lee, R.J. Taylor, Y.K. Tam, C.L. Hansen, P.R. Cullis, Microfluidic synthesis of highly potent limit-size lipid nanoparticles for in vivo delivery of siRNA, *Mol. Ther. Nucleic Acids* in press.

[218] D.D. Stuart, G.Y. Kao, T.M. Allen, A novel long-circulating and functional liposomal formulation of antisense oligonucleotides targeted against MDR1, *Cancer Gene Ther.* 7 (2000) 466–475.

[219] G. Pagnan, D.D. Stuart, F. Pastorino, L. Raffaghello, P.G. Montaldo, T.M. Allen, B. Calabretta, M. Ponzoni, Delivery of c-myc antisense oligodeoxynucleotides to human neuroblastoma cells via disialoganglioside GD2-targeted immunoliposomes: antitumor effects, *J. Natl. Cancer Inst.* 92 (2000) 253–261.

[220] F. Pastorino, C. Brignole, D. Marimpietri, G. Pagnan, A. Morando, D. Ribatti, S.C. Semple, C. Gambini, T.M. Allen, M. Ponzoni, Targeted liposomal c-myc antisense oligodeoxynucleotides induce apoptosis and inhibit tumor growth and metastases in human melanoma models, *Clin. Cancer Res.* 9 (2003) 4595–4605.

[221] C. Brignole, F. Pastorino, D. Marimpietri, G. Pagnan, A. Pistorio, T.M. Allen, V. Pistoia, M. Ponzoni, Immune cell-mediated antitumor activities of GD2-targeted liposomal c-myc antisense oligonucleotides containing CpG motifs, *J. Natl. Cancer Inst.* 96 (2004) 1171–1180.

[222] S.D. Li, L. Huang, Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells, *Mol. Pharm.* 3 (2006) 579–588.

[223] D. Di Paolo, C. Brignole, F. Pastorino, R. Carosio, A. Zorzoli, M. Rossi, M. Loi, G. Pagnan, L. Emionite, M. Cilli, S. Bruno, R. Chiarle, T.M. Allen, M. Ponzoni, P. Perri, Neuroblastoma-targeted nanoparticles entrapping siRNA specifically knockdown ALK, *Mol. Ther.* 19 (2011) 1131–1140.

[224] D. Di Paolo, C. Ambrogio, F. Pastorino, C. Brignole, C. Martinengo, R. Carosio, M. Loi, G. Pagnan, L. Emionite, M. Cilli, D. Ribatti, T.M. Allen, R. Chiarle, M. Ponzoni, P. Perri, Selective therapeutic targeting of the anaplastic lymphoma kinase with liposomal siRNA induces apoptosis and inhibits angiogenesis in neuroblastoma, *Mol. Ther.* 19 (2011) 2201–2212.

[225] D.C. Litzinger, J.M. Brown, I. Wala, S.A. Kaufman, G.Y. Van, C.L. Farrell, D. Collins, Fate of liposomes and their complex with oligonucleotide in vivo, *Biochim. Biophys. Acta* 1281 (1996) 139–149.

[226] D.D. Stuart, T.M. Allen, A new liposomal formulation for antisense oligodeoxynucleotides with small size, high incorporation efficiency and good stability, *Biochim. Biophys. Acta* 1463 (2000) 219–229.

[227] N. Maurer, A. Mori, L. Palmer, M.A. Monck, K.W. Mok, B. Mui, Q.F. Akhong, P.R. Cullis, Lipid-based systems for the intracellular delivery of genetic drugs, *Mol. Membr. Biol.* 16 (1999) 129–140.

[228] D.E. Lopes de Menezes, Y. Hu, L.D. Mayer, Combined treatment of Bcl-2 antisense oligodeoxynucleotides (G3139), p-glycoprotein inhibitor (PSC833), and sterically stabilized liposomal doxorubicin suppresses growth of drug-resistant growth of drug-resistant breast cancer in severely combined immunodeficient mice, *J. Exp. Ther. Oncol.* 3 (2003) 72–82.

[229] F. Pastorino, C. Brignole, D. Di Paolo, B. Nico, A. Pezzolo, D. Marimpietri, G. Pagnan, F. Piccardi, M. Cilli, R. Longhi, D. Ribatti, A. Corti, T.M. Allen, M. Ponzoni, Targeting liposomal chemotherapy via both tumor cell-specific and tumor vasculature-specific ligands potentiates therapeutic efficacy, *Cancer Res.* 66 (2006) 10073–10082.

[230] L.D. Mayer, T.O. Harasym, P.G. Tardi, N.L. Harasym, C.R. Shew, S.A. Johnstone, E.C. Ramsay, M.B. Bally, A.S. Janoff, Ratiometric dosing of anticancer drug combinations: controlling drug ratios after systemic administration regulates therapeutic activity in tumor-bearing mice, *Mol. Cancer Ther.* 5 (2006) 1854–1863.

[231] F. Pastorino, D.R. Mumbengegwi, D. Ribatti, M. Ponzoni, T.M. Allen, Increase of therapeutic effects by treating melanoma with targeted combinations of c-myc antisense and doxorubicin, *J. Control. Release* 126 (2008) 85–94.

[232] A. Dicko, L.D. Mayer, P.G. Tardi, Use of nanoscale delivery systems to maintain synergistic drug ratios in vivo, *Expert Opin. Drug Deliv.* 7 (2010) 1329–1341.

[233] M. Loi, S. Marchiò, P. Becherini, D. Di Paolo, M. Soster, F. Curnis, C. Brignole, G. Pagnan, P. Perri, I. Caffa, R. Longhi, B. Nico, F. Bussolino, C. Gambini, D. Ribatti, M. Cilli, W. Arap, R. Pasqualini, T.M. Allen, A. Corti, M. Ponzoni, F. Pastorino, Combined targeting of perivascular and endothelial tumor cells enhances anti-tumor efficacy of liposomal chemotherapy in neuroblastoma, *J. Control. Release* 145 (2010) 66–73.

[234] N.R. Patel, A. Rath, D. Mongayt, V.P. Torchilin, Reversal of multidrug resistance by co-delivery of tariquidar (XR9576) and paclitaxel using long-circulating liposomes, *Int. J. Pharm.* 416 (2011) 296–299.

[235] J.M. Hare, T.M. Allen, Combined anti-vasculature and anti-tumor targeting in the treatment of metastatic breast cancer, in: 36th Annual Meeting of the Controlled Release Society, 2009.

[236] E.A. Moase, A.T.M., Combination targeting of immunoliposomal therapeutics in a murine model of human B lymphoma, in: 36th Annual Meeting of the Controlled Release Society, 2009.

[237] M. Saad, O.B. Garbuzenko, T. Minko, Co-delivery of siRNA and an anticancer drug for treatment of multidrug-resistant cancer, *Nanomedicine (Lond.)* 3 (2008) 761–776.

[238] Y. Chen, J.J. Wu, L. Huang, Nanoparticles targeted with NGR motif deliver c-myc siRNA and doxorubicin for anticancer therapy, *Mol. Ther.* 18 (2010) 828–834.

[239] K. Un, S. Kawakami, R. Suzuki, K. Maruyama, F. Yamashita, M. Hashida, Enhanced transfection efficiency into macrophages and dendritic cells by a combination method using mannosylated lipoplexes and bubble liposomes with ultrasound exposure, *Hum. Gene Ther.* 21 (2010) 65–74.

[240] E.J. Feldman, J.E. Lancet, J.E. Kolitz, E.K. Ritchie, G.J. Roboz, A.F. List, S.L. Allen, E. Asatiani, L.D. Mayer, C. Swenson, A.C. Louie, First-in-man study of CPX-351: a liposomal carrier containing cytarabine and daunorubicin in a fixed 5:1 molar ratio for the treatment of relapsed and refractory acute myeloid leukemia, *J. Clin. Oncol.* 29 (2011) 979–985.

[241] <http://www.celatorpharma.com/new/products.html>.

[242] J.H. Ryu, H. Koo, I.C. Sun, S.H. Yuk, K. Choi, K. Kim, I.C. Kwon, Tumor-targeting multi-functional nanoparticles for theragnosis: new paradigm for cancer therapy, *Adv. Drug Deliv. Rev.* (2012) July 4 (Epub ahead of print).

[243] T.M. Allen, P.R. Cullis, Drug delivery systems: entering the mainstream, *Science* 303 (2004) 1818–1822.

[244] J.R. Morgan, L.A. Williams, C.B. Howard, Technetium-labelled liposome imaging for deep-seated infection, *Br. J. Radiol.* 58 (1985) 35–39.

[245] C.A. Presant, R.T. Proffitt, A.F. Turner, L.E. Williams, D. Winsor, J.L. Werner, P. Kennedy, C. Wiseman, K. Gala, R.J. McKenna, J.D. Smith, S.A. Bouzalou, R.A. Callahan, J. Baldeschwieler, R.J. Crossley, Successful imaging of human cancer with Indium-111-labelled phospholipid vesicles, *Cancer* 62 (1988) 905.

[246] C.A. Presant, D. Blayney, R.T. Proffitt, A.F. Turner, L.E. Williams, H.I. Nadel, P. Kennedy, C. Wiseman, K. Gala, R.J. Crossley, et al., Preliminary report: imaging of Kaposi sarcoma and lymphoma in AIDS with indium-111-labelled liposomes, *Lancet* 335 (1990) 1307–1309.

[247] Y. Matsumura, H. Maeda, A new concept for macromolecular therapeutics in cancer chemotherapy; mechanism of tumoritropic accumulation of proteins and the antitumor agent SMANCS, *Cancer Res.* 46 (1986) 6387–6392.

[248] K.J. Harrington, S. Mohammadtaghi, P.S. Uster, D. Glass, A.M. Peters, R.G. Vile, J.S. Stewart, Effective targeting of solid tumors in patients with locally advanced cancers by radiolabeled pegylated liposomes, *Clin. Cancer Res.* 7 (2001) 243–254.

[249] A. Bochot, E. Fattal, Liposomes for intravitreal drug delivery: a state of the art, *J. Control. Release* 161 (2012) 628–634.

[250] N.M. Bressler, V.I.P.T.S. Group, Verteporfin therapy of subfoveal choroidal neovascularization in age-related macular degeneration: two-year results of a randomized clinical trial including lesions with occult and no classic choroidal neovascularization – verteporfin in photodynamic therapy report 2, *Am. J. Ophthalmol.* 131 (2001) 541–560.

[251] A. Schroeter, T. Engelbrecht, R.H. Neubert, A.S. Goebel, New nanosized technologies for dermal and transdermal drug delivery. A review, *J. Biomed. Nanotechnol.* 6 (2010) 511–528.

[252] C. Gómez, M. Benito, J.M. Teijón, M.D. Blanco, Novel methods and devices to enhance transdermal drug delivery: the importance of laser radiation in transdermal drug delivery, *Ther. Deliv.* 3 (2012) 373–388.

[253] M.T. Krauze, C.O. Noble, T. Kawaguchi, D. Drummond, D.B. Kirpotin, Y. Yamashita, E. Kullberg, J. Forsayeth, J.W. Park, K.S. Bankiewicz, Convection-enhanced delivery of nanoliposomal CPT-11 (irinotecan) and PEGylated liposomal doxorubicin (Doxil) in rodent intracranial brain tumor xenografts, *Neuro Oncol.* 9 (2007) 393–403.

[254] J.P. Motion, G.H. Huynh, F.C.J. Szoka, R.A. Siegel, Convection and retroconvection enhanced delivery: some theoretical considerations related to drug targeting, *Pharm. Res.* 28 (2011) 472–479.

[255] I.M. Hann, H.G. Prentice, Lipid-based amphotericin B: a review of the last 10 years of use, *Int. J. Antimicrob. Agents* 17 (2001) 161–169.

[256] R.N. Davidson, L. Di Martino, L. Gradioti, R. Giachino, R. Russo, G.B. Gaeta, R. Pempinello, S. Scott, F. Raimondi, A. Cascio, et al., Liposomal amphotericin B (AmBisome) in Mediterranean visceral leishmaniasis: a multi-centre trial, *Q. J. Med.* 87 (1994) 75–81.

[257] F. Muggia, J. Hainsworth, S. Jeffers, S. Groschen, M. Tan, F.A. Greco, Liposomal doxorubicin (Doxil®) is active against refractory ovarian cancer, *Proc. Am. Soc. Clin. Oncol.* 15 (1996) 287.

[258] J. Bladé, P. Sonneveld, J.F. San Miguel, H.J. Sutherland, R. Hajek, A. Nagler, A. Spencer, T. Robak, K.C. Lantz, S.H. Zhuang, J.L. Harousseau, R.Z. Orlowski, D.-M.-S. Investigator, Efficacy and safety of pegylated liposomal Doxorubicin in combination with bortezomib for multiple myeloma: effects of adverse prognostic factors on outcome, *Clin. Lymphoma Myeloma Leuk.* 11 (2011) 44–49.

[259] Y. Barenholz, Doxil® – the first FDA-approved nano-drug: lessons learned, *J. Control. Release* 160 (2) (2012) 117–134.

[260] C.E. Petre, D.P. Dittmer, Liposomal daunorubicin as treatment for Kaposi's sarcoma, *Int. J. Nanomedicine* 2 (2007) 277–288.

[261] G. Batist, G. Ramakrishnan, C.S. Rao, A. Chandrasekharan, J. Gutheil, T. Guthrie, P. Shah, A. Khojasteh, M.K. Nair, K. Hoelzer, K. Tkaczuk, Y.C. Park, L.W. Lee, Reduced cardiotoxicity and preserved antitumor efficacy of liposome-encapsulated doxorubicin and cyclophosphamide compared with conventional doxorubicin and cyclophosphamide in a randomized, multicenter trial of metastatic breast cancer, *J. Clin. Oncol.* 19 (2001) 1444–1454.

[262] R. Bowden, P. Chandrasekar, M.H. White, X. Li, L. Pietrelli, M. Gurwith, J.A. van Burik, M. Laverdiere, S. Safrin, J.R. Wingard, A double-blind, randomized, controlled trial of amphotericin B colloidal dispersion versus amphotericin B for treatment of invasive aspergillosis in immunocompromised patients, *Clin. Infect. Dis.* 35 (2002) 359–366.

[263] T.J. Walsh, J.W. Hiemenz, N.L. Seibel, J.R. Perfect, G. Horwith, L. Lee, J.L. Silber, M.J. DiNubile, A. Reboli, E. Bow, J. Lister, E.J. Anaissie, Amphotericin B lipid complex for invasive fungal infections: analysis of safety and efficacy in 556 cases, *Clin. Infect. Dis.* 26 (1998) 1383–1396.

[264] D. Gambling, T. Hughes, G. Martin, W. Horton, G. Manvelian, A comparison of Depodur, a novel, single-dose extended-release epidural morphine, with standard epidural morphine for pain relief after lower abdominal surgery, *Anesth. Analg.* 100 (2005) 1065–1074.

[265] M.J. Glantz, S. LaFollette, K.A. Jaekle, W. Shapiro, L. Swinnen, J.R. Rozental, S. Phuphanich, L.R. Rogers, J.C. Gutheil, T. Batchelor, D. Lyter, M. Chamberlain, B.L. Maria, C. Schiffer, R. Bashir, D. Thomas, W. Cowens, S.B. Howell, Randomized trial of a slow-release versus a standard formulation of cytarabine for the intrathecal treatment of lymphomatous meningitis, *J. Clin. Oncol.* 17 (1999) 3110–3116.

[266] M.J. Glantz, K.A. Jaekle, M.C. Chamberlain, S. Phuphanich, L. Recht, L.J. Swinnen, B. Maria, S. LaFollette, G.B. Schumann, B.F. Cole, S.B. Howell, A randomized controlled trial comparing intrathecal sustained-release cytarabine (DepoCyt) to intrathecal methotrexate in patients with neoplastic meningitis from solid tumors, *Clin. Cancer Res.* 5 (1999) 3394–3402.

[267] M.R. Patrick, I.J. Blair, R.O. Feneck, P.S. Sebel, A comparison of the haemodynamic effects of propofol ('Diprivan') and thiopentone in patients with coronary artery disease, *Postgrad. Med. J.* 61 (1985) 23–27.

[268] J.A. Simon, E.S. Group, Estradiol in micellar nanoparticles: the efficacy and safety of a novel transdermal drug-delivery technology in the management of moderate to severe vasomotor symptoms, *Menopause* 13 (2006) 222–231.

[269] http://www.tlcbio.com/en/product_lipo_dox.html.

[270] A.H. Sarris, F. Hagemeyer, J. Romaguera, M.A. Rodriguez, P. McLaughlin, A.M. Tsimerman, L.J. Medeiros, B. Samuels, O. Pate, M. Ohlendorf, H. Kantarjian, C. Burge, F. Cabanillas, Liposomal vincristine in relapsed non-Hodgkin's lymphomas: early results of an ongoing phase II trial, *Ann. Oncol.* 11 (2000) 69–72.

[271] M.A. Rodriguez, R. Pytlak, T. Kozak, M. Chanahabai, R. Gascoyne, B. Lu, S.R. Deitcher, J.N. Winter, M. Investigators, Vincristine sulfate liposomes injection (Marqibo) in heavily pretreated patients with refractory aggressive non-Hodgkin lymphoma: report of the pivotal phase 2 study, *Cancer* 115 (2009) 3475–3482.

[272] M.S. Newman, G.T. Colbern, P.K. Working, C. Engbers, M.A. Amantea, Comparative pharmacokinetics, tissue distribution, and therapeutic effectiveness of cisplatin encapsulated in long-circulating, pegylated liposomes (SPI-077) in tumor-bearing mice, *Cancer Chemother. Pharmacol.* 43 (1999) 1–7.

[273] N. Seetharamu, E. Kim, H. Hochster, F. Martin, F. Muggia, Phase II study of liposomal cisplatin (SPI-77) in platinum-sensitive recurrences of ovarian cancer, *Anticancer Res.* 30 (2010) 541–545.

[274] K. Riviere, H.M. Kieler-Ferguson, K. Jerger, F.C.J. Szoka, Anti-tumor activity of liposome encapsulated fluorouracil acid as a single agent and in combination with liposome irinotecan, *J. Control. Release* 153 (2011) 288–296.

[275] <http://www.celatorpharma.com/new/products.html>.

[276] C. McDonagh, A. Huhalov, B. Harms, S. Adams, V. Paragas, O. S., B. Zhang, L. Luus, R. Overland, S. Nguyen, J. Gu, N. Kohli, M. Wallace, M. Feldhaus, A. Kudla, B. Schoeberl, U. Nielsen, Antitumor activity of a novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3, *Mol. Cancer Ther.* 11 (3) (2012) 582–593.

[277] <http://www.talontx.com/pipeline.php?divid=brakiva>.

[278] <http://www.talontx.com/pipeline.php?divid=alocrest>.

[279] M. Fantini, L. Gianni, C. Santelmo, F. Drudi, C. Castellani, A. Affatato, M. Nicolini, A. Ravaioli, Lipoplatin treatment in lung and breast cancer, *Chemother. Res. Pract.* 2011 (2011) 125192.

[280] G.P. Stathopoulos, D. Antoniou, J. Dimitroulis, J. Stathopoulos, K. Marosis, et al., Comparison of liposomal cisplatin versus cisplatin in non-squamous cell non-small-cell lung cancer, *Cancer Chemother. Pharmacol.* 68 (2011) 945–950.

[281] E. Apostolidou, R. Swords, Y. Alvarado, F.J. Giles, Treatment of acute lymphoblastic leukaemia: a new era, *Drugs* 67 (2007) 2153–2171.

[282] D.J. Booser, F.J. Esteve, E. Rivera, V. Valero, L. Esparza-Guerra, W. Priebe, G.N. Hortobagyi, Phase II study of liposomal annamycin in the treatment of doxorubicin-resistant breast cancer, *Cancer Chemother. Pharmacol.* 50 (2002) 6–8.

[283] R.T. Poon, N. Borys, Lysophosphatidylserine liposomal doxorubicin: an adjuvant to increase the cure rate of radiofrequency ablation in liver cancer, *Future Oncol.* 7 (2011) 937–945.

[284] R. Staruch, R. Chopra, K. Hynnen, Localised drug release using MRI-controlled focused ultrasound hyperthermia, *Int. J. Hyperthermia* 27 (2011) 156–171.

[285] U. Fasol, A. Frost, M. Büchert, J. Arends, U. Fiedler, D. Scharr, J. Scheuenpflug, K. Mross, Vascular and pharmacokinetic effects of EndoTAG-1 in patients with advanced cancer and liver metastasis, *Ann. Oncol.* 23 (2012) 1030–1036.

[286] <http://www.tekmirapharm.com/Programs/Products.asp>.

[287] P.A. Bradbury, F.A. Shepherd, Immunotherapy for lung cancer, *J. Thorac. Oncol.* 3 (2008) S164–S170.

[288] B.M. Richard, P. Newton, L.R. Ott, D. Haan, A.N. Brubaker, P.J. Cole, P.E. Ross, M.C. Rebelatto, K.G. Nelson, The safety of EXPAREL® (bupivacaine liposome injectable suspension) administered by peripheral nerve block in rabbits and dogs, *J. Drug Deliv.* 2012 (2012) 962101.

[289] G. Berry, M. Billingham, E. Alderman, P. Richardson, F. Torti, B. Lum, A. Patek, F.J. Martin, The use of cardiac biopsy to demonstrate reduced cardiotoxicity in AIDS Kaposi's sarcoma patients treated with pegylated liposomal doxorubicin, *Ann. Oncol.* 9 (1998) 711–716.

[290] T. Safra, F. Muggia, S. Jeffers, D.D. Tsao-Wei, S. Groshen, O. Lyass, R. Henderson, G. Berry, A. Gabizon, Pegylated liposomal doxorubicin (Doxil): reduced clinical cardiotoxicity in patients reaching or exceeding cumulative doses of 500 mg/m², *Ann. Oncol.* 11 (2000) 1029–1033.

[291] J.J. Lokich, C. Moore, Chemotherapy-associated palmar-plantar erythrodysesthesia syndrome, *Ann. Intern. Med.* 101 (1984) 798–800.

[292] P.G. Rose, Pegylated liposomal doxorubicin: optimizing the dosing schedule in ovarian cancer, *Oncologist* 10 (2005) 205–214.